An investigation into the environmental drivers of evolution in marine predators

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An investigation into the environmental drivers of evolution in marine predators

By

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For Emily

The Ocean's greatest ally.

Abstract

Understanding the key environmental drivers of population structure formation and evolution is a fundamental problem in evolutionary biology. Within marine environments, ocean frontal systems have been implicated in acting as a barrier to gene flow in a wide variety of marine taxa, yet their impact on population structure in marine predators remains unclear.

Using a combination of genetic markers (SNPS and mtDNA loci) and stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) this study investigated the genetic and trophic population structure of the Yellowmouth Barracuda *Sphyraena viridensis* and the Bottlenose Dolphin *Tursiops truncatus* against the context of environmental variables. *S. viridensis* showed some limited evidence of geographic population structure but considerable evidence of differential feeding by geography and the presence of two clear haplogroups. By contrast, clear population structure was evident in *T. truncatus* with the Almería-Oran front presenting as a strong environmental influence. Further environmental factors (e.g. salinity) correlated with population structure, with its impact on prey distribution being a possible causal mechanism. Evidence for an offshore Azores-Sicily metapopulation of *T. truncatus* was discovered, possibly mediated by social and acoustic parameters, although data on this is limited.

There was clear genetic differentiation between *T. truncatus* ecotypes but with evidence of ongoing gene flow. A possible sequence of events to explain gene flow patterns within the wider genus is explored. Investigations in to the timing of speciation between *T. truncatus* and *T. aduncus* revealed evidence for climate events being a key driver of evolution in this genus. This builds on previous evidence that climate is a fundamentally important driver in the evolution of cetaceans.

Declaration

The material contained within this thesis has not previously been submitted for a degree at Durham University or any other university. The research reported here has been conducted by the author unless stated otherwise.

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General Introduction

1.1 Evolution and the environment

In its broadest sense evolution is the generational change in heritable traits found in living organisms (Barton *et al*., 2007). Although it is now widely accepted within the scientific community that evolution is responsible for the full suite of diversity found in life on Earth, the drivers of this process are not yet fully understood. In particular, understanding how environmental gradients and boundaries drive the formation of genetic population structure, divergence and ultimately speciation remains a fundamental challenge in evolutionary biology.

1.1.1 The terrestrial realm and the cradle of understanding

Most scientific examinations of the influence of environment on population structure and gene flow have focussed on terrestrial species (Sork and Waits, 2010). This is not surprising as, compared to the marine environment, terrestrial habitats are relatively accessible and less expensive to study. As a result, a great deal of our understanding of the environmental influences on population structure is largely derived from terrestrial ecosystems. Many of these terrestrial studies exemplify core concepts in the environmental influences of evolutionary biology and are directly transferrable to the marine environment. There are two main environmental features to be considered: barriers (physical features that represent the sharp transition between one environment and another) and gradients (clinal variations in environmental factors

that can be either steep or shallow but also represent transitions between environments).

When considering physical barriers to gene flow aquatic features can pose as much a barrier to terrestrial species gene flow as land can to marine species. For example, during the last several periods of glaciation deep water channels maintained the Wallacea region of Indonesia as an island archipelago. These seaways created a significant gene flow barrier to many terrestrial species, leading to a high level of endemism as a result of allopatric speciation. Bats have been a focus of many studies in the region as they are abundant and exhibit exceptionally high levels of endemism for mammals (Campbell *et al*., 2007; Hisheh *et al*., 2000; Schmitt *et al*., 2009). Schmitt *et al.* (1995) examined allozymes, as a proxy of genetic variation, in fruit bats found at 20 islands across the Wallacea region and found that genetic distance correlated not only to modern sea-crossing distances between islands but also strongly with estimated sea crossings at the time of the last glacial maximum. This study clearly demonstrates that the sea can form a significant barrier to gene flow for terrestrial species, even ones that we think of as being highly mobile. It is important to note here that in the case of bats it is likely the differential distribution of suitable food resources between islands that drives genetic differentiation, rather than an impassable barrier to movement.

However, aquatic barriers to gene flow need not be marine. Numerous studies have shown that even the relatively short distance of the width of rivers can limit gene flow (Gascon *et al*., 2000; Peres *et al*., 1996; Vallinoto *et al*., 2006). In a study of 19 species of non-volant mammals on the island of Borneo by Brunke *et al.* (2019) it was found that for some species the Kinabatangan river in Sabah represented an absolute barrier to gene flow and the population on either riverbank was in total genetic

isolation from the other. Even volant species, such as birds, have been shown to have reduced gene flow between populations as a result of rivers (Fernandes *et al*., 2014).

Physical barriers can be in the form of features of the land. For example, Zalewski *et al.* (2009) demonstrated in their study of American Mink (*Neovision vison*) in Scotland that the Cairngorm mountain range represented a significant impediment to gene flow in the region. Physical barriers can sometimes come in a slightly more surprising and anthropogenic form. In studies of plant species the Great Wall of China has been found to be a significant gene flow restrictor, keeping populations separate for over 600 years (Su *et al*., 2003). Roads too have been noted for their ability to restrict gene flow in terrestrial species (Epps *et al*., 2005; Keller and Largiadèr, 2003; Riley *et al*., 2006). However, it is rare, except for examples such as vicariance brought about tectonic continental division, for barriers to be absolute. More often they offer limited gene flow rather than a complete blockade, as in most of the examples presented above.

Our planet is very rarely a patchwork of one habitat abutted against another, more often it is a gradual transition between each one. Much like the temporal defining of a species, habitats work on a sliding scale window with an almost infinite possibility of defining characteristics. With this in mind, when trying to understand drivers of population structure researchers should be alert to the influence of gradients and not look immediately to obvious environmental features.

Environmental gradients can have peculiar impacts when it comes to gene flow and genetic differentiation between populations, particularly in relation to clinal steepness of the gradient in question. For example, it has been shown that along a smooth environmental gradient sharp differences in genotype may appear

spontaneously and are not indicative of a corresponding sharp environmental change at the steepest genetic cline (Endler, 1973). By this mechanism, environmental gradients have the ability to create differentiation within, and ultimately between, populations sympatrically (Doebeli and Dieckmann, 2005).

The steepness of any environmental gradient matters considerably. It is recognised that environmental gradients can contribute to overall fitness in a species by maintaining an optimum level of gene flow between populations (Alleaume-Benharira *et al*., 2006). If gene flow is too low between populations this can result in heterogeneity of overall species fitness as local adaptation can reduce diversity and the population propensity for phenotypic plasticity. However, if gene flow is too high between populations then this can lead to gene swamping where immigrants can inhibit local genetic adaptation (Lenormand, 2002; Polechová and Barton, 2015).

In a meta-analysis of 70 independent studies of gene flow in environmental gradients, Sexton *et al.* (2014) showed that a pattern of isolation by environment (IBE) whereby gene flow was highest between environments of similar parameters was the most common scenario, surpassing either isolation by distance (IBD) or counter-gradient gene flow (greater gene flow between disparate environments). From this one can expect that, for highly mobile animals at least, that gene flow is most likely between similar habitats even if it means the individuals passing potential mates in more dissimilar habitats *en route*.

Environmental gradients can also have an indirect effect on gene flow within a species by affecting its prey. There are many examples of intraspecies specialisation in prey choice (Dickman and Newsome, 2015; Robertson *et al*., 2014; Sheppard *et al*., 2018). When a predator's population range overlaps the limit of a prey species range,

restricted by environmental factors, then this can lead to a dichotomy in prey specialisation within the predator population, in some cases leading to a restriction in gene flow and genetic divergence. Terrestrial prey specialisation resulting in population divergence is particularly well documented in Arachnida (Pekár and Toft, 2015).

These studies clearly illustrate that gene flow, and thus population structure, of a species can be heavily influenced by environment. However, all of the cited examples so far come from the terrestrial environment or relate to volant fauna. Next, I consider whether these principles are directly transferrable to the marine environment or whether there are other factors at play in influencing gene flow and population structure in marine organisms.

1.2 Evolution in the marine environment

1.2.1 Marine vs terrestrial environments

There are numerous key differences between the marine and terrestrial environment that might influence a species population structure. The first major difference between the oceans and the terrestrial biome is that the oceans are a fully three-dimensional environment. The dimension of depth brings with it numerous environmental gradients including light, pressure and primary productivity (Boyle, 1660; Falkowski *et al*., 1998; Lorenzen, 1972).

Even when we only consider the properties of the ocean near-surface, as this thesis shall do, they still present a complex habitat. It is tempting to consider our oceans as a continuous and uninterrupted environment, with none of the harsh barriers

to gene flow like the examples we considered in the terrestrial biome. However, the waters of the world's oceans are not continuous but rather form discrete water masses, each with their own water properties and environmental variables. The meeting of two water masses, in what is known as an ocean front, forms a steep environmental cline that may form some element of obstacle. Like on land, within ocean basins or within water masses there can be environmental gradients too, particularly of factors like sea surface temperature (SST) or salinity, that often run along longitudinal or latitudinal axes.

1.2.2 Marine environmental drivers of evolution

As in terrestrial systems, hard physical barriers are the most easily understood barriers to gene flow. In the marine realm, landmasses form the most obvious physical barriers, principally in the form of continents but also peninsulas and isthmuses. By far the most studied example of this is the Isthmus of Panama. This Isthmus formed approximately 2.8 million years ago and the closure of the preceding seaway initiated the flow of the Gulf Stream. A large number of species exhibit genetic divergence across this barrier, with the evidence suggesting that there were once high levels of gene flow before its formation. Examples of which range from seahorses (*Hippocampus erectus*) (Boehm *et al*., 2013) to flying fish (*Exocoetus volitans*) (Lewallen *et al*., 2016). Despite being only 50km wide, this isthmus is known as a major barrier to gene flow in a number of seabird species, even ones that are known to make significant at-sea migrations (Avise *et al*., 2000; Morris-Pocock *et al*., 2016, 2011; Steeves *et al*., 2005). Like with the Isthmus of Panama, signatures of historical gene flow supporting geological evidence for historically passable seaways has been seen in other locations too, such as the Isthmus of Kra in southeast Asia (de Bruyn *et*

al., 2005). Sea ice can also form a barrier akin to landmasses and has been suggested to isolate the North Atlantic and North Pacific Humpback Whale *Megaptera novaeangliae* populations (Ruegg *et al*., 2013) as well as being a movement barrier to the Gray Whale *Eschrichtius robustus* (McKeon *et al*., 2016). Climate change and the consequent melting of permanent sea ice may test this theory.

Whilst submarine mountains and seamounts may facilitate gene flow for many marine species (Shank, 2010; Vecchione *et al*., 2010), the deep open water which surrounds them often acts as a significant barrier for species with life histories that include a planktonic or pelagic juvenile stage to their life history where such an environment would present an increased risk of predation (Portnoy *et al*., 2014). A notable example of this is the East Pacific Barrier, an area of deep water approximately 5000km across that separates the eastern and central Pacific, which has been recognised as a genetic barrier to many marine species (Duncan *et al*., 2006; Schultz *et al*., 2008). Interestingly, a number of marine species with apparently similar life histories or ecologies, have exhibited substantial gene flow across this barrier including species of reef fish (Lessios *et al*., 1998; Lessios and Robertson, 2006) and sharks (Clarke *et al*., 2015) thus showing that impact generalisations are to be avoided.

Ovenden *et al.* (2009) conducted a study of the population structure of four species of sharks with notable ecological similarity found within Australian and Indonesian waters as well as into the Indian Ocean using microsatellite and mitochondrial markers and found that whilst deep trenches could be responsible as a barrier in some species it was certainly not the case for all. The authors are explicit in their statement that ecological similarity is not necessarily a predictor of similar patterns of gene flow and that species-specific studies are often needed to understand patterns of connectivity. This approach will contribute towards the finding of

commonalities in environmental drivers of evolution across taxa, a fundamental aim of molecular ecology.

In many cases it is improbable that a single environmental factor is the sole restrictor of gene flow for a given species. Portnoy *et al*. (2014) undertook a study of the population structure of Blacknose Shark *Carcharhinus acronotus* in the Caribbean and up the eastern seaboard of the United States using mitochondrial and microsatellite markers. In this study it was suggested that a genetic break between those sharks found along the eastern coast and the southern coast (and the Bahamas) was as a result of not only a deep water channel but also the Florida peninsula and the strong currents of the Florida straits, something also shown for other species (Avise, 1992; Gold *et al*., 2009, 2002). This examination by Portnoy *et al*. (2014) was also suggestive that in other regions of the study area further population structure was created by other potential barriers to gene flow such as a narrowing in the continental shelf edge, something that would be limiting for a species relying on shallow water to hunt.

There are several examples of ocean currents, presenting as frontal systems, acting as barriers to gene flow in marine megafauna. It has been shown that the warm waters of the Gulf Stream form a significant barrier to gene flow between north Atlantic populations of the Tope Shark *Galeorhinus galeus* (Chabot and Allen, 2009). It is the opposite effect with the Whale Shark *Rhincodon typus,* for whom studies have shown that it is the cold water currents of the Benguela current that could be limiting gene flow between the Atlantic and Indian oceans as prolonged exposure to cold water can be fatal in this species (Beckley *et al*., 1997; Castro *et al*., 2007).

It is important to note that in both of these cases it is the differential in temperature that is likely the main driver of differentiation, the currents are merely the

driver of this differential. Sharp differences in temperature (and/or salinity) between marine water bodies, or ocean fronts, are well documented as being linked to forming population structure within species. In a study of European Perch *Perca fluviatilis* within the Baltic sea, it was found that the largest genetic break was coincident with the steepest gradient in spring water temperatures (Bergek *et al*., 2010). Similarly, studies have found strong correlations between population structure and geographical changes in both water temperature and salinity in Atlantic Herring *Clupea harengus* (Bekkevold *et al*., 2005; Jorgensen *et al*., 2005) and Atlantic Cod *Gadus morhua* (Nielsen *et al*., 2009). Although ocean frontal systems coincide with population boundaries in the majority of these studies environmental gradients have also been implicated as a potential driver of population structure formation (Hemmer-Hansen *et al*., 2007). This thesis will attempt to examine the influence of oceanic fronts and, to a lesser extent, environmental gradients on the population structure of marine megafauna species, in this case in the Mediterranean Sea.

1.3 Environmental echoes; can environments leave a deeper impression?

Advances in sequencing technology have provided us with the ability to examine whole genomes of organisms (Morozova and Marra, 2008). This presents the opportunity to examine not only the current process of adaptation as in the studies highlighted so far, but also examples of historical adaptation and even historical speciation as driven by temporal environmental changes. Through analysis of genomic data of closely related species it is possible to elucidate divergence times (Cahill *et al*., 2016). Where these divergences coincide with known environmental changes or the

opening of new environmental niches, genuine questions can be asked if there is a causal link between such events and species radiation and divergence (Kim *et al*., 2014; Li *et al*., 2014). Such comparisons have been made in marine species (Kishida, 2017; Vijay *et al*., 2018) but this thesis proposes to take this further by first examining admixture since divergence and also looking to see if the environmental influences that potentially caused speciation in the first place can be studied by comparison to a contemporary proxy.

1.4 The Mediterranean Sea

The Mediterranean Sea (Figure 1.1) is a significant body of water that lies between the African and European continents, joining the Atlantic Ocean on its extreme western margin through the Strait of Gibraltar and bordered by the Asian continent on its eastern edge. More specifically, it lies in the region between longitudes 6°W and 36°E and latitudes 30° and 46°N. The area occupied by the Mediterranean Sea is approximately 2,510,00 square kilometres, with its dimensions running for approximately 4000km from east to west (Gulf of Iskenderun to Strait of Gibraltar) and 800km from north to south (northern Italy to Libya).

Until 1869 the Mediterranean Sea's only real connection to other bodies of water was to the Atlantic Ocean through the Strait of Gibraltar and to the Black Sea through the Dardanelles. This relative isolation has led to the development of a unique ecosystem and area for study. In 1869 the completion of the Suez Canal connected the Mediterranean to the Red Sea and the Indian Ocean beyond; the subsequent invasion of non-native species into the Mediterranean region is well studied and is a significant

area of concern for environmentalists (Coll *et al*., 2010; Molnar *et al*., 2008; Rilov and Galil, 2009).

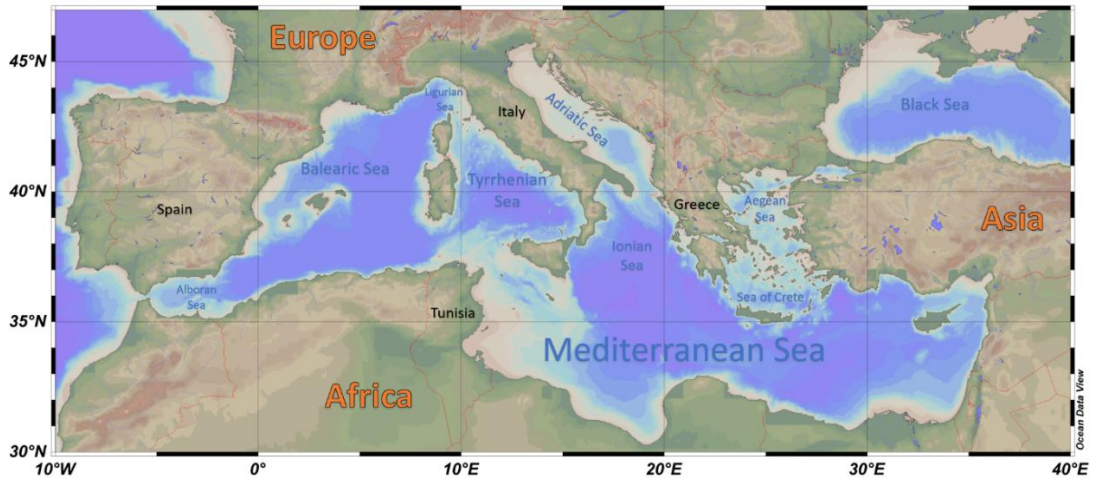

Figure 1.1: A map of the Mediterranean Sea with key sea regions identified. Map created using *Ocean Data View*

1.4.1 Contemporary physical aspects

The Mediterranean Sea is divided into separate regions both physically and oceanographically. The principal division separates the Eastern Basin from the Western Basin and comprises both physical and oceanographic differences. A submarine sill (a rise in the ocean floor) in the Sicily Channel (comprising the Adventure Plateau, Malta Platform and Tunisia Platform – see Figure 1.3C), with a mean depth of about 360 metres, running between the Italian island of Sicily in the north to the African coast of Tunisia in the south separates the two main basins. This physical delineation is accompanied by an oceanographic front, commonly known as the Siculo-Tunisian front (STF) that runs along a similar axis for some of the year, though at times developing significantly more complex characteristics. This frontal system separates warmer and more saline water to the south-east and cooler, fresher water to the north-west. The STF operates on a seasonal cycle, being stronger and

more defined in the summer and autumn months and reducing in strength in the months of winter and into spring (Figure 1.2).

Each of these main basins is then sub-divided into smaller basins, most notably by sills. The Eastern Basin of the Mediterranean Sea is comprised of two main basins and a number of small seas. The Levantine Basin comprises the region south of Turkey and north of Egypt, delineated along its western edge by the island of Crete and a submarine ridge that runs between Crete and Libya. The second main basin in the Eastern Mediterranean is the Ionian Basin which lies to the east of the Sicily Channel sill, west of the island of Crete and south of Greece. The Ionian Basin contains the deepest area of the Mediterranean Sea with a maximum depth of approximately 4,900 metres. Further regions of the Eastern Mediterranean Sea include the Aegean Sea, found in the relatively shallow area north of Crete and populated by the most islands of any Mediterranean region, and the Adriatic Sea which lies between Italy in the west and the Balkan states to the east. The Adriatic Sea forms an almost perfectly linear environmental gradient of warmer, shallower water in the north and cooler, deeper water in the south.

The Western Mediterranean Basin can be subdivided into three main regions; The Tyrrhenian Basin, the Algerian Basin and the Alborán Basin. The Tyrrhenian Basin, containing the Tyrrhenian Sea, is in the region west of Italy and east of the islands of Corsica and Sardinia. Although relatively deep, the Tyrrhenian Basin is characterised by a large number of seamounts including 14 whose peak is sufficiently shallow to allow colonisation by photosynthetic organisms (Bo *et al*., 2011). The Algerian Basin is the largest basin in the Western Mediterranean and lies to the west of Corsica and Sardinia, containing the Balearic Sea. Finally, the relatively small and shallow Alborán Basin sits between southern Spain in the north and Morocco to the

south. The delineation between the Alborán and Algerian Basins is also accompanied by an oceanographic frontal system – the Almería-Oran Front (AOF) (Figure 1.4). The AOF runs approximately between the city of Almería in Andalusia, southern Spain and the city of Oran in north-western Algeria. The Alborán basin contains two gyres, the West Alborán Gyre and the East Alborán Gyre; the AOF forms the easternmost boundary of the East Alborán Gyre. The AOF is similar to the Siculo-Tunisian Front in that it separates fresher water in the west from more saline water to the east but in contrast to the Siculo-Tunisian Front it is present year-round with no significant drop in strength during spring months.

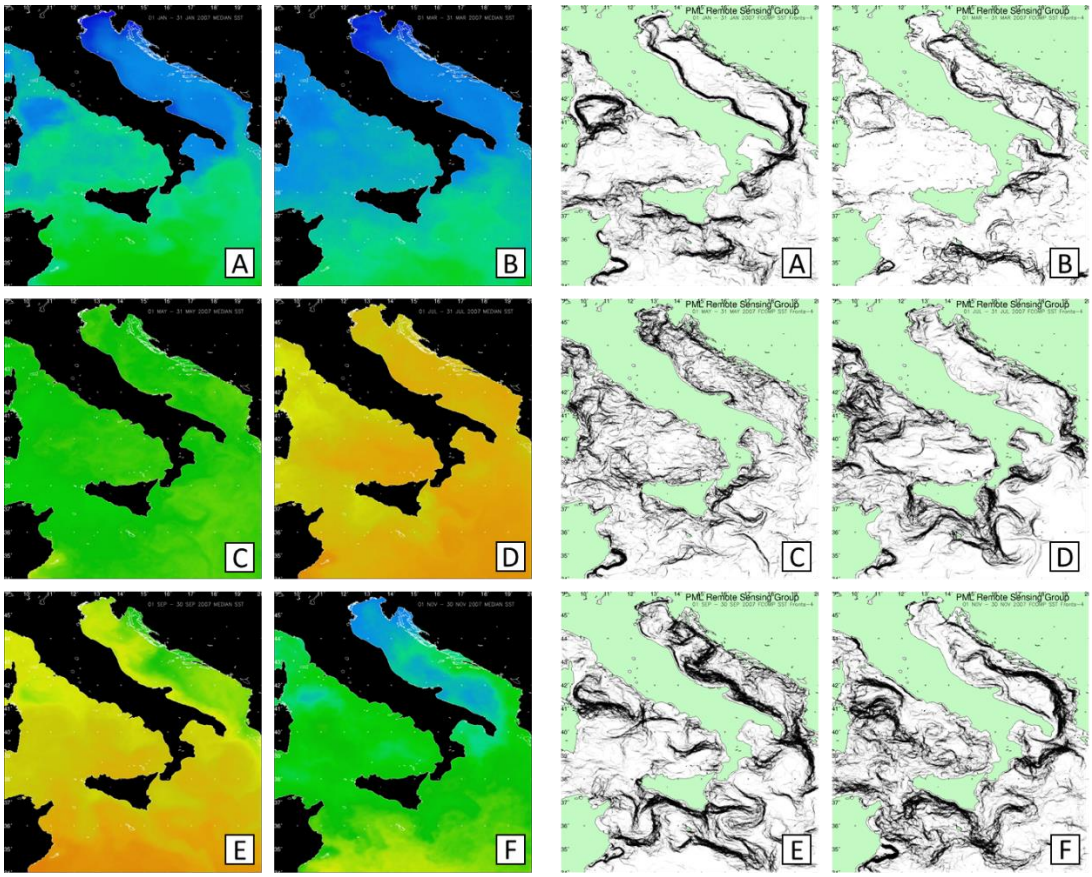

Figure 1.2: Seasonal satellite imagery of Sea Surface Temperature (SST) around Italy (Left) clearly showing seasonal variation. Righthand images depict formation of frontal systems and depict the variation in strength and complexity of the Siculo-Tunisian front system off the south coast of Sicily. Images are taken through the year 2007 – A (January), B (March), C (May), D (July), E (September) and F (November). All data and images © Plymouth Marine Laboratory Remote Sensing Group.

Sea Surface Temperatures (SST) across the Mediterranean vary significantly (Figure 1.3A). The highest SST is found in Libyan waters (Gulf of Sidra) where mean values for August peak around 31°C. Lowest SSTs are found in the northern Adriatic where in winter temperatures can reach as low as 5°C, for comparison the winter temperatures of surface waters off the Mediterranean coast of Egypt rarely fall below 17°C (Said et al., 2007). Generally higher SST values are seen in the Eastern Basin than the Western Basin, mainly due to its slightly lower latitude. Deeper waters hold far more consistent temperatures, with all water below a depth of about 900 metres holding year-round temperatures of approximately 13°C.

Relative to other large bodies of water the Mediterranean Sea has high salinity, reaching a peak of 40 parts per thousand during the peak of summer in the Eastern Mediterranean Basin (Figure 1.3B). Freshwater input to the Mediterranean Sea from rivers equals only about 30% of the total volume of water that is lost to evaporation (the remaining 70% comes from Atlantic marine input via the Gibraltar Strait) thus resulting in the high salinity levels. This major imbalance of water input to output drives the main circulatory features of the Mediterranean: the surface water inflow from the Atlantic Ocean through the Gibraltar Strait. This surface water current flows eastward and although weaker is still apparent in the Eastern Mediterranean Basin. This surface current forms two large anti-clockwise eddies, one in each basin, and constitutes the main hydrographic features of the Mediterranean Sea. The current is significantly stronger during the summer months, when evaporation rates are at their highest. During the higher air temperatures of the summer months, when evaporation rates are at their highest, the surface waters of the Mediterranean Sea become denser due to the increase in salinity. This causes the denser surface water to sink and in so doing, forming the denser layer of bottom water. As this bottom layer builds any

excess vents into the Atlantic Ocean over the sill in the Strait of Gibraltar, flowing below the inward surface current and forming a counter-current system in this region.

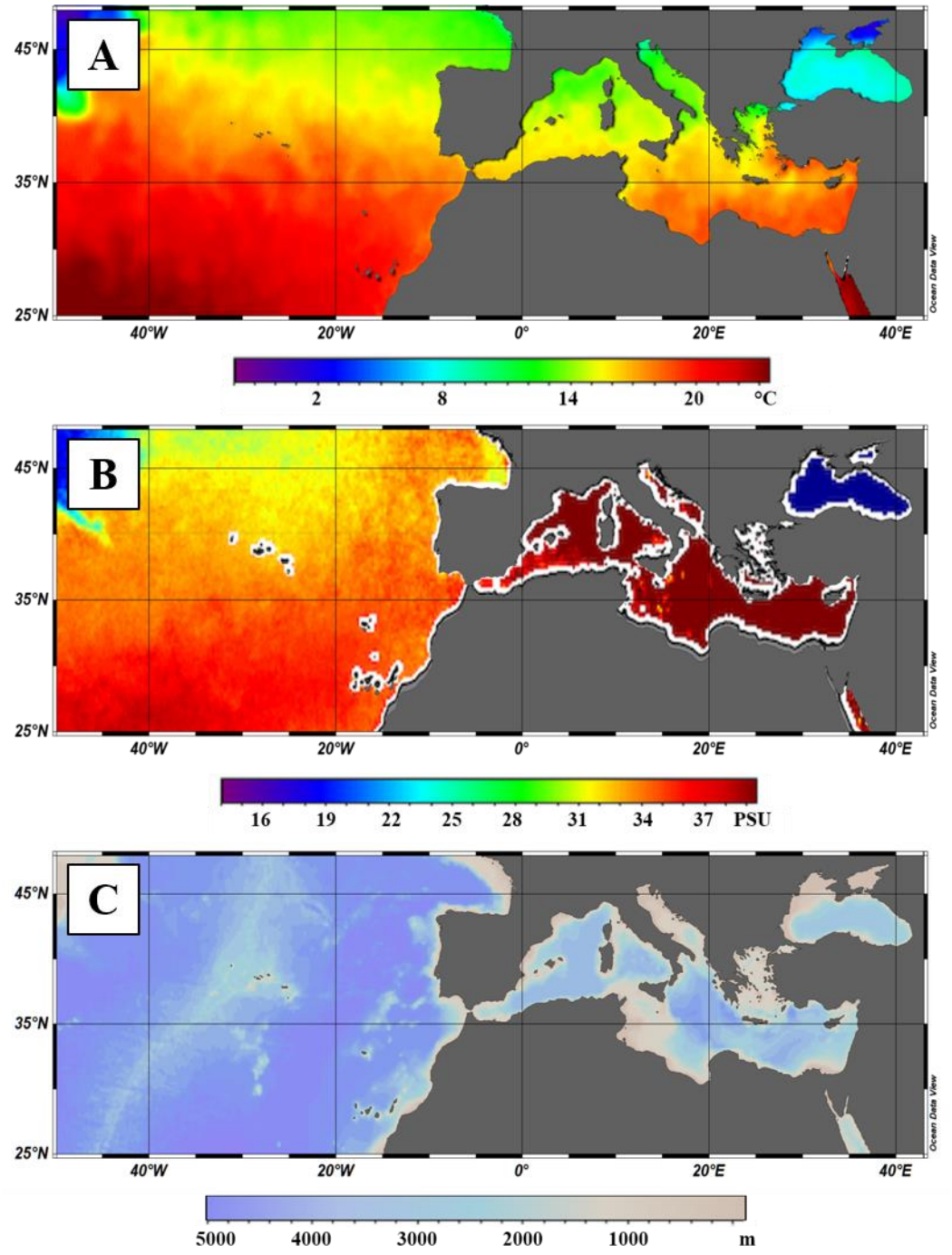

Figure 1.3: There is considerable oceanographic variation across the Mediterranean and eastern Atlantic. Panel A presents the mean summer SST, Panel B the mean annual salinity and Panel C the depth of water.

There are several major current systems in the Mediterranean (see Figure 1.5), many of which are important in the context of this thesis. One of the strongest currents in the Mediterranean is the Algerian Current, transforming in to the Atlantic-Ionian Stream as it passes Sicily, that transports water from the Atlantic eastwards into the eastern basin. This current contours the north African coast and passes over the Sicily sill into the Ionian. Also notable is the western basin gyre that is comprised of several smaller current systems, firstly departing northward from the Algerian current in to the Tyrrhenian Gyre before following an anti-clockwise path around the western Mediterranean along the Ligurian Current and the Northern Current before returning eastwards along the Balearic Current.

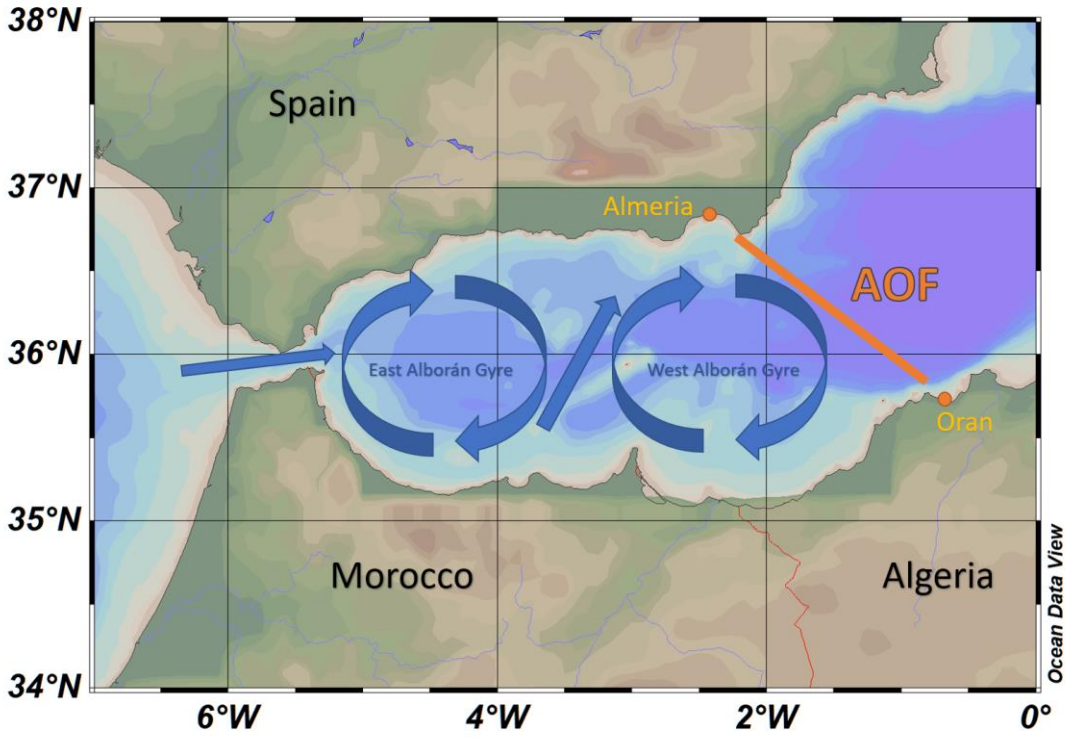

Figure 1.4: The Almería-Oran front (AOF) at the eastern margin of the Alborán basin is depicted here by the solid orange line. The two gyres of the Alborán basin are depicted by the dark blue circular arrows. Map created using *Ocean Data View*

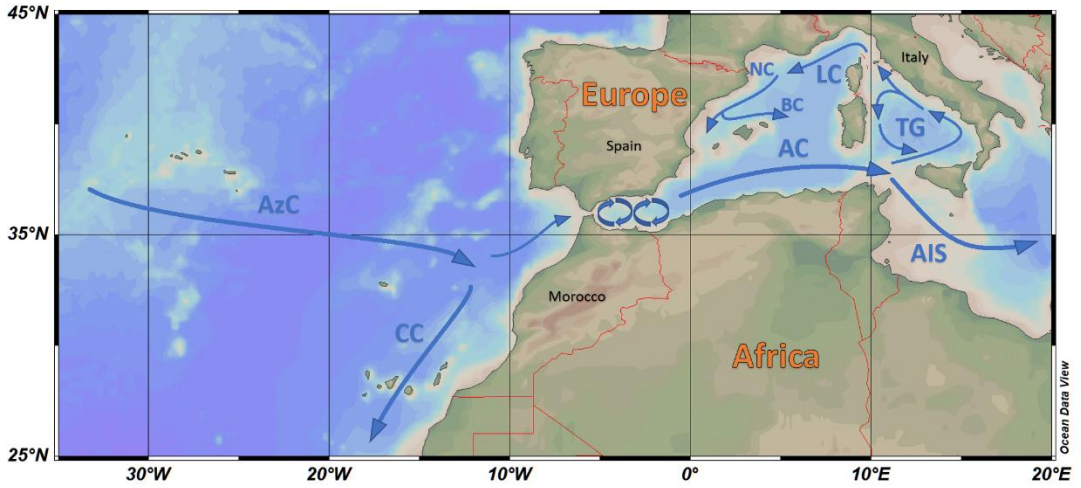

Figure 1.5: Major current systems in the Mediterranean and eastern Atlantic pertinent to this thesis. Consisting of the Azores Current (AzC), Canary Current (CC), Algerian Current (AC), Atlantic-Ionian Stream (AIS), Tyrrhenian Gyre (TG), Ligurian Current (LC), Northern Current (NC) and Balearic Current (BC).

1.4.2 History

It was once thought that the Mediterranean derived directly from the remnants of the Tethys Ocean, the vast ancient sea that separated the Eurasian and African plates and ran from modern Spain in the west to Nepal and China in the East. However, modern understanding of plate tectonics and seabed cores that demonstrate the crust below the Mediterranean is relatively recent have shown that this is not the case (Hsü, 1977; Laubscher and Bernoulli, 1977). Current understanding suggests that as the African plate pushed northwards into Eurasia and the Tethys Ocean was consumed, the resulting tectonic upheaval created a number of smaller basins, particularly in the eastern region. It is thought that one of these new basins, named the Neotethys, expanded throughout the Cenozoic to become the modern Mediterranean Sea that exists today.

All extant marine megafauna in the Mediterranean, and the ecosystems they inhabit, must have become established from outside sources and only within the last 5 million years. This is because around six million years ago, in what is termed the Messinian Salinity Crisis, the Mediterranean Basins consisted only of low lying and highly saline lakes (Roveri *et al*., 2014) around 3000 metres below modern sea level. This was caused by a period of strong Antarctic glaciation that dropped sea levels and formed a land bridge running across the modern-day Strait of Gibraltar (Ohneiser *et al*., 2015). The Messinian Salinity Crisis is likely to have had a profound effect on the evolution of Mediterranean marine life – essentially restarting the clock as the remaining bodies of water, although deep in places, were likely so saline that they were inhospitable to large marine life and this should be kept in mind when consideration is given to the origins of the genus *Tursiops* in later chapters. The Messinian Salinity Crisis came to an end around 5.33 million years ago when the Atlantic Ocean breached the Gibraltar land bridge in an event known as the Zanclean Flood (Garcia-Castellanos *et al*., 2009). Although the mechanism and speed of this breach is still the subject of intense scientific debate it is thought that the Mediterranean likely attained near modern-day sea levels in around 2 years (Micallef *et al*., 2018).

Throughout the Quaternary the cyclic pattern of ice ages had a noticeable impact on the Mediterranean Sea that likely brought great challenges to the marine life which inhabited it. It is known from sediment cores that, during periods of glaciation in some areas of the Mediterranean Sea surface water temperatures were up to 6°C cooler than the present day (Hayes *et al*., 2005) and accompanied by a $2.7^{0}/_{00}$ rise in salinity (Thunell and Williams, 1989). Post-glacial periods saw a subsequent crash in salinity of up to $5^{0}/_{00}$ as a result of the formation of great glacial meltwater lakes that

formed on the Eurasian continent and that subsequently drained into the basin (Thunell, 1979). Furthermore, the cyclic rise and fall of sea levels during the Pleistocene would have globally repeatedly decreased the shallow coastal habitat available before expanding it again. Dependent on local slope gradient, this would have forced some marine megafauna species, who specialise in coastal environments or prey species, in to deeper waters; potentially being a key driver in cetacean evolution that is explored further in Chapter 4.

1.4.3 Fauna

It is estimated that there are around 12,000 macroscopic species to be found in the Mediterranean (Boudouresque, 2004). It is estimated that around 20% of these species are endemic (Deidun, 2011) and it is principally for this reason that the Mediterranean is considered as a global hotspot for marine biodiversity (Bianchi and Morri, 2000).

The Mediterranean is an oligotrophic (low available nutrients) sea and consequentially has low levels of primary productivity (Turley *et al*., 2000). The oligotrophic nature of the Mediterranean is driven by its low levels of dissolved nitrates and phosphates, a status brought about by the input of already oligotrophic water from the eastern Atlantic and by the very limited number of large rivers that drain into either of its main basins. However, all is not even and the eastern Mediterranean is considerably more oligotrophic than the western Mediterranean, with a steady gradient of falling primary production from west to east (Psarra *et al*., 2000). The result of this is that, despite its great diversity, the Mediterranean will never

achieve the great biomass of the more productive seas of north-western Europe (Chassot *et al*., 2007; Lloret *et al*., 2006).

The Mediterranean is largely microtidal (that is its tides have an amplitude less than 2m), a feature that is mostly determined, apart from a few regional exceptions, by its relatively limited continental shelf area. As such the intertidal zone in the Mediterranean is very limited and certainly does not feature the species diversity or biomass found in some other coastal areas of the world with greater tidal range.

It has substantial areas of deep-water environments, typically classified as those areas greater than 200m deep (the average depth of water in the Mediterranean is just under 1500m), and spatially these represent the greatest available biome for marine life. The deepest recorded point within the Mediterranean is within the Calypso Deep; found within the Ionian Sea, it was recorded as being 5,267m deep. Danovaro *et al*. (2010) estimated that approximately 2805 macrofaunal species could be found within the deep waters of the Mediterranean and that of these 66% remain to be discovered. However, the greatest species biodiversity is still to be found in the coastal waters of the Mediterranean and with over 46,000 km of coastline this is no surprise.

The Mediterranean is also an important habitat for predatory fish species including many of the large Scombridae such as the Bluefin Tuna *Thunnus thynnus*, Albacore Tuna *Thunnus alalonga* and the Skipjack Tuna *Katsuwonis pelamis*. Other predatory fish present in the Mediterranean include at least 47 shark species (including the White Shark *Carcharodon Carcharias* (Morey *et al*., 2003)), Swordfish *Xiphias gladius*, Common Dolphinfish *Coryphaena hippurus* and six species of Barracuda (*Sphyraena* spp.).

Marine mammals also feature extensively in the Mediterranean species list with at least 29 species known to occur; 12 of these are seen regularly and 17 species have been recorded from occasional sightings (di Sciara, 2016). Seven of the 12 species regularly seen in the Mediterranean Sea are listed as threatened on the IUCN Red List, which leads to a mention of the species that, by far, has the biggest impact on the Mediterranean environment and its fauna.

The Mediterranean Sea is one of the most industrialised marine areas in the world and *Homo sapiens* have, almost universally, negatively impacted population numbers of many of its species. From water pollution (Karydis and Kitsiou, 2012), to overfishing (Papaconstantinou and Farrugio, 2000), noise pollution (Notarbartolo-di-Sciara *et al*., 2008) and of course plastics (Wai Chin *et al*., 2016), humans have undoubtedly placed pressures on all species living here. It currently remains to be seen if, taken as any other environmental pressure, marine species are able to adapt to cope with some of these relatively new anthropogenic challenges and if the differential application of these pressures has an impact on inter-population gene flow.

1.5 East-West Mediterranean divergence and other barriers to gene flow

As introduced in section 1.4.1 the Mediterranean can be defined as two distinct basins, the West Mediterranean and the East Mediterranean, separated by the channel between the Italian shores of Sicily and Tunisia known as the Siculo-Tunisian Strait. At its narrowest the Siculo-Tunisian Strait, defined oceanographically by the Siculo-Tunisian front (STF), is 155 kilometres wide and so hardly presents a barrier to gene flow on first appearance. However, numerous population genetics studies (presented

below) have shown this to be the case for a wide variety of taxa, as well as revealing other potential oceanographic barriers to gene flow. It is important to note here that there is often a conflict of findings based on the number and types of genetic markers used (Gharbi *et al*., 2011, 2010), thus giving a strong mandate for the application of high-resolution NGS methodologies applied in this thesis.

Studies on crustacea appear particularly prevalent. Zitari-Chatti *et al.* (2007) made investigations into the genetic structure of the Caramote Prawn *Paneus kerathurus* in the Siculo-Tunisian region using 13 allozyme loci and found a significant (F_{ST} = 0.076, P<0.05) differentiation in populations concurrent with a two basin structure. Similarly, Deli *et al.* (2015) found that there was strong genetic differentiation between Eastern and Western Mediterranean localities along the coast of Tunisia for the Green Crab *Carcinus aestuarii* (F_{ST} = 0.535, P<0.001) when using the mitochondrial DNA (mtDNA) marker COI. Interestingly, the authors of this study note that there was no correlation of differentiation and geographic distances but that there was corresponding phenotypic traits suggestive of adaptation to differing environmental conditions found in the two basins (Said *et al*., 2014). East-West differentiation in *C. aestuarii* is supported by a variety of previous studies (Marino *et al*., 2011; Ragionieri and Schubart, 2013). Yet we should not be misled into thinking that similar patterns could be expected for all crustacea – Fratini *et al.* (2016) investigated the genetic population structure of *Pachygrapsus marmoratus* also using the mtDNA marker COI and 6 microsatellite markers and found no evidence for differentiation between Mediterranean basins. The deep-sea environment also appears to offer little resistance to gene flow for crustacean species with a study on the deep-sea crustacean *Aristeus atennatus* showing high levels of gene flow and relative panmixia throughout the Mediterranean (Maggio *et al*., 2009).

Mollusca, too, have been subject to examination of their genetic population structure in the Mediterranean Sea and these studies also tell a very mixed story. Tassinari (2012) examined the Banded Murex, a marine gastropod, *Hexaplex trunculus* using the mtDNA marker COI and found significant genetic differentiation between the East and West coasts of Italy (F_{ST} = 0.257, P=0.000), delineated by the STF. This is supported by later studies (Marzouk *et al*., 2016) that showed similar significant differentiation across the STF. There is conflicting interpretations in the study of the Carpet Shell Clam *Ruditapes decussatus* (Gharbi *et al*., 2011, 2010) whereby mtDNA (COI) and ITS1 analysis showed no significant differentiation either side of the STF but use of 15 allozyme loci did. Like the crustaceans, the significance of the STF as a factor in determining population structure in the mollusca is not clear as there are examples appearing to show homogeneity across the region. For example, the razor clam *Solen marginatus* showed no genetic alignment with the STF but only more localised differentiation (Hmida *et al*., 2012) though the limited scale of this study should be noted. On a more appropriate scale Sanna *et al.* (2013) examined the Pen Shell *Pinna nobilis* to find significant genetic structuring across the Mediterranean Sea using two mtDNA markers (COI & 16S). Interestingly the identified populations in this study were not concordant with an East-West paradigm that is aligned with the STF but is split further East and the authors go on to suggest that biogeographic boundaries and Pleistocene changes in sea levels are the likely drivers of this structuring. This inference is derived from evidence of eastward expansion and a later founder event in the Aegean, a geographical region not covered in many of the other studies mentioned here. Other oceanographic features have also been demonstrated to be significant barriers to gene flow for Mollusca with the Almería-Oran front separating the Atlantic and Mediterranean populations of the Common Cuttlefish,

Sepia officinalis (Pérez-Losada *et al*., 2002), a pattern also observed in the mussel *Mytilus galloprovincialis* (El Ayari *et al*., 2019; Quesada *et al*., 1995) and scallop species *Pecten jacobaeus* and *P. maximus* (Rios *et al*., 2002).

Studies of the Echinodermata are few but at least one reveals the STF as a barrier to gene flow and transition between populations in the Red Comb Star *Astropecten arancicus* (Zulliger *et al*., 2009). The east-west divergence present in many species is not only restricted to Animalia, we have seen a similar genetic structure in the seagrass *Posidonia oceanic* too (Arnaud-Haond *et al*., 2007; Serra *et al*., 2010).

The picture becomes more confused when we consider the Vertebrata, particularly those known to be highly mobile or that make significant migrations. Carreras *et al*. (2006) examined the population structure of immature Loggerhead Turtle, *Caretta caretta,* across the Mediterranean using the mtDNA control region (CR) and found that structure aligned with ocean currents rather than fronts or bathymetric features, particularly in the western basin whereby individuals found in the north, along the coast of Europe, grouped with those of the eastern Mediterranean. By contrast, those in the south, along the African coast, shared haplotypes with known Atlantic populations. This latitudinal split in haplotypes replicates the dichotomous current system of the Algerian Current in the south and the Ligurian-Northern Currents in the north. It is likely that the location spent during early life stages of loggerhead turtles, the so-called ‘lost years’, are heavily influenced by such current systems and form the basis for this population structure.

The population structure of numerous fish species has been examined in the Mediterranean. Schunter *et al.* (2011a) conducted a study of the Dusky Grouper

Epinephelus marginatus using microsatellites and although no oceanographic barrier was found they did find a very similar genetic structure to that found for Loggerhead Turtles by Carreras *et al.* (2006) which suggests that the Dusky Grouper may also be influenced strongly by Mediterranean current systems. In the same year Schunter *et al.* (2011a) also examined the Comber *Serranus cabrilla* using 11 microsatellite markers and although they found no differentiation at the Siculo-Tunisian front they did find that the Almería-Oran front presented a significant barrier to gene flow with further restrictions caused by the Balearic Front and the Ibiza Channel. The Almería-Oran front has now been shown to a significant barrier to gene flow for numerous fish species including the European Bass *Dicentrarchus labrax* (Naciri *et al*., 1999), European Hake *Merluccius merluccius* (Cimmaruta *et al*., 2005), Two Banded Bream *Diplodus vulgaris*, Peacock Wrasse *Symphodus tinca* and Striped Red Mullet *Mullus surmuletus* (Galarza *et al*., 2009).

In a study of the Spotted Catshark *Scyliorhinus canicula* it was found the STF was a restrictor on gene flow between the two Mediterranean basins, although the authors stress that some of this genetic structuring may be a result of past glacial-era oceanography rather than contemporary features and that further work was needed (Kousteni *et al*., 2015). The STF has been found to be a barrier to gene flow in a number of other fish species including the Mackerel *Scomber scombrus* (Zardoya *et al*., 2004), Common Sole *Solea solea* (Rolland *et al*., 2007), European Hake *Merluccius merluccius* (Cimmaruta *et al*., 2005) and the Gilthead Bream *Sparus aurata* (De Innocentiis *et al*., 2004) (with further sub-divisions).

Bluefin Tuna *Thunnus thynnus* present a particular curiosity when it comes to east-west divergence as there is a great deal of conflict between studies, thus a true understanding remains elusive. Some studies support a Siculo-Tunisian restriction in

gene flow (Boustany *et al*., 2008; Carlsson *et al*., 2004) whilst others do not (Bremmer *et al*., 2005; Pujolar *et al*., 2003; Viñas *et al*., 2011).

Despite the various fronts and current systems presenting genetic barriers to many fish species there still remains some that appear to exist with high gene flow between sub-groups and thus with only a single population in the Mediterranean such as the Swordfish *Xiphias gladius* (Pujolar *et al*., 2002). However, this study also suggested there was low-level evidence of divergence at the STF and that further work is needed,

Within the Cetacea studies are somewhat limited, particularly for the larger species. An examination of the Striped Dolphin *Stenella coeruleoalba* using five microsatellite markers revealed genetic differentiation between Mediterranean and Atlantic populations but sample paucity means the exact barrier to gene flow could not be identified. The authors do not resolve clear structure within the Mediterranean (Bourret *et al*., 2007) but a higher resolution re-examination of this species with a greater number of markers has revealed strong evidence for an East-West Mediterranean divergence (Gkafas *et al*., 2017). This interpretation is supported by an earlier study that coincides the population break with the STF (Gaspari *et al*., 2007), something also observed in Short-Beaked Common Dolphin *Delphinus delphis* (Natoli *et al*., 2008).

Lack of population structure across the Mediterranean has been suggested for the Sperm Whale *Physeter macrocephalus* (Drouot *et al*., 2004), which is supported by observations of inter-basin movements by individuals (Frantzis *et al*., 2011) but more recent examination with NGS methodologies is finding some evidence of a bi-

population structure, though with a north-south division rather than alignment with the STF (Violi *et al*., 2019).

One cetacean species that has received a good amount of examination, at least regionally, is the Bottlenose Dolphin *Tursiops truncatus*. It has been suggested that the STF is a significant barrier to gene flow in this species (Natoli *et al*., 2005). Other regional studies have shown levels of population substructure within the Mediterranean and neighbouring areas (Fernández *et al*., 2011b; Gaspari *et al*., 2015a, 2015b) but further research is needed to fully understand the overall population structure and its corresponding environmental drivers.

An interesting example of environmental barriers to gene flow in Cetacea is that of the Harbour Porpoise *Phocoena phocoena* where there is strong genetic divergence between the northeast Atlantic population and the Black Sea population (Fontaine *et al*., 2007), something also seen in *T. truncatus* (Moura *et al*., 2013). Harbour Porpoises are found in cool waters of the northern hemisphere but are nearly absent from the Mediterranean Sea (except a presence in the Aegean Sea (see Cucknell *et al*., 2016)), which now forms a very large barrier to gene flow between the aforementioned populations. However, they were once believed to exist here when its waters were significantly cooler around 7000 years ago, thus allowing the colonisation of the Black Sea from the Atlantic population (Frantzis *et al*., 2001)

The Mediterranean as a whole is undoubtedly a longitudinal basin (Tanhua *et al*., 2013) and given the distance it spans and the dispersal capabilities of many species overviewed in this section, isolation by distance (IBD) cannot be ruled out as a potential driver of divergence. However, given that physical environmental conditions are so strong and locally variable it is likely that they have a significant role to play.

Table 1.1: Summary (non-exhaustive) of relevant literature on genetic divergence patterns observed in the Mediterranean. N= number of individuals used in study. GF = Gene Flow. GF barriers (where not defined) are STF = Siculo-Tunisian Front, AOF = Almerían-Oran Front, BF = Balearic Front, GT = Gulf of Trieste & IC = Ibiza Channel. Studies of Bluefin Tuna *Thunnus thynnus* have been omitted due to the conflict in agreement, please see main text for details.

Species	**Common name**	**Phylum**	**Distribution**	**Study scale**	**Genetic marker**	**N**	**GF barrier**	**References**
Melicertus kerathurus	Caramote Prawn	Arthropoda	E Atl & Med	Local	Allozymes	287	STF	Zitari-Chatti *et al.* (2008)
Carcinus aestuarii	Green Crab	Arthropoda	Med	Local	Mitochondrial (COI)	88	STF	Deli *et al.* (2015)
Carcinus aestuarii	Green Crab	Arthropoda	Med	Med	Mitochondrial (COX1)	199	STF	Ragioneri & Schubart (2013)
Carcinus aestuarii	Green Crab	Arthropoda	Med	Regional	Mitochondrial (COI)	596	STF	Marino *et al.* (2011)
Pachygrapsus marmoratus	Marbled Rock Crab	Arthropoda	E Atl & Med	Med	Mitochondrial (COI) & Microsatellites	587	None	Fratini *et al.* (2016)
Aristeus atennatus	Red Shrimp	Arthropoda	E Atl, Med & Indian O.	Regional	Mitochondrial	175	None	Maggio *et al.* (2009)
Hexaplex trunculus	Banded Murex	Mollusca	E Atl & Med	Regional	Mitochondrial (COI)	240	STF	Tassinari *et al.* (2012)
Ruditapes decussatus	Grooved Carpet Shell	Mollusca	E Atl & Med	Local	Mitochondrial (COI), rDNA (ITS-1) & Allozymes	170	STF	Gharbi *et al.* (2010,2011)
Solen marginatus	Grooved Razor Shell	Mollusca	E Atl & Med	Local	Allozymes	189	None	Hmida *et al.* (2012)

Pinna nobilis	Fan Mussel	Mollusca	Med	Med	Mitochondrial (COI & 16S)	236	STF, Otranto Str. & Cretian Pass.	Sanna *et al.* (2013)
Caretta caretta	Loggerhead Turtle	Chordata	Cosmopolitan	Regional	Mitochondrial (D-loop)	282	Balearic-Sardinia	Carreras *et al.* (2006)
Serranus cabrilla	Comber	Chordata	E Atl & Med	Med	Microsatellites	382	AOF, BF & IC	Schunter *et al.* (2011a)
Epinephelus marginatus	Dusky Grouper	Chordata	E Atl & Med, SW Atl	Med	Microsatellites	362	Balearic-Sardinia	Schunter *et al.* (2011b)
Scyliorhinus canicula	Spotted Catshark	Chordata	E Atl & Med	Med	Mitochondrial (COI)	431	STF	Kousteni *et al.* (2015)
Posidonia oceanica	Neptune Grass	Tracheophyta	Med	Local	Microsatellites	560, 1,360	STF	Serra *et al.* (2010), Arnaud-Haond *et al.* (2007)
Astropecten arancicus	Red Comb Star	Echinodermata	E Atl & Med	Med	Mitochondrial	254	STF	Zulliger *et al.* (2009)
Sepia officinalis	Common Cuttlefish	Mollusca	Atl, Med & Baltic	Local	Microsatellites	439	AOF	Pérez-Losada *et al.* (2002)
Pecten jacobaeus/ P. maximus	Mediterranean/Great Scallop	Mollusca	Med	Regional	Allozymes	1398	AOF	Rios *et al.* (2002)
Mytilus galloprovincialis	Mediterranean Mussel	Mollusca	E Atl & Med	Med	Mitochondrial	Unk, 568.	AOF	Quesada *et al.* (1995), El Ayari *et al.* (2019)
Dicentrarchus labrax	European Bass	Chordata	E Atl & Med	Regional	Microsatellites	630	AOF	Naciri *et al.* (1999)

Scomber scombrus	Mackerel	Chordata	N Atl, Med & Baltic	Med	Mitochondrial (CR)	285	STF	Zardoya *et al.* (2004)
Xiphias gladius	Swordfish	Chordata	Cosmopolitan	Med	Allozymes	401	None	Pujolar *et al.* (2002)
Solea solea	Common Sole	Chordata	E Atl & Med	Med	Nuclear (EPIC)	749	STF	Rolland *et al.* (2006)
Sparus aurata	Gilthead Sea Bream	Chordata	E Atl & Med	Med	Microsatellites	361	STF, Corsica-Sardinia	De Innocentiis *et al.* (2004)
Diplodus vulgaris	Two Banded Bream	Chordata	E Atl & Med	Regional	Microsatellites	190	AOF	Galarza *et al.* (2009)
Mullus surmuletus	Red Striped Mullet	Chordata	E Atl & Med	Regional	Microsatellites	192	AOF	Galarza *et al.* (2009)
Symphodus tinca	Peacock Wrasse	Chordata	E Atl & Med	Regional	Microsatellites	195	AOF	Galarza *et al.* (2009)
Stenella coeruleoalba	Striped Dolphin	Chordata	Cosmopolitan	Med	Microsatellites	137	AOF/Gibraltar Str.	Bourret *et al.* (2007)
Merluccius merluccius	European Hake	Chordata	E Atl & Med	Med	Allozymes	1306	AOF, STF	Cimmaruta *et al.* (2005)
Stenella coeruleoalba	Striped Dolphin	Chordata	Cosmopolitan	Med	Microsatellites	165	STF	Gaspari *et al.* (2007)
Physeter macrocephalus	Sperm Whale	Chordata	Cosmopolitan	Med	Mitochondrial (CR)	57	None	Drouot *et al.* (2004)
Delphinus delphis	Short-Beaked Common Dolphin	Chordata	Tropical Cosmopolitan	Med	Microsatellites	118	STF	Natoli *et al.* (2008)

Tursiops truncatus	Common Bottlenose Dolphin	Chordata	Cosmopolitan	Med	Microsatellites & Mitochondrial (CR)	145	STF	Natoli *et al.* (2005)
Tursiops truncatus	Common Bottlenose Dolphin	Chordata	Cosmopolitan	Regional	Microsatellites & Mitochondrial (CR)	89	Otranto Str., GT & Cretian Pass	Gaspari *et al.* (2015a)
Tursiops truncatus	Common Bottlenose Dolphin	Chordata	Cosmopolitan	Regional	Microsatellites & Mitochondrial (CR)	194	Otranto Str., GT & Cretian Pass	Gaspari *et al.* (2015b)

1.6 Can the marine environment act as a driver of evolution in marine predators?

It has been clearly demonstrated in the previous examination of literature that the environment is a key driver in evolution of marine species, including highly mobile predators. However, it is not immediately clear which environmental factors or features are likely to be key for a given species.

A quick examination of Table 1.1 reveals the variation of gene flow barriers across taxa. However, there may be some confounding of clarity due to the scope, scale and methods employed by the presented studies. A high-resolution examination of population structure in multiple species that also considers environmental and feeding ecology across the same geographical area may allow us to develop a deeper understanding of the environmental drivers that have a common evolutionary impact across taxa. Next-Generation Sequencing technologies would allow a much higher resolution study of population structure than any study featured in Table 1.1.

Furthermore, an examination of the genomes of two species, thought to be diverged by an environmental driver, would provide insight in to the mechanism of environmentally driven speciation and its lasting impression on a species genome. A particular area of interest would be to see how complete speciation is and to what level admixture occurs after species divergence. Admixture is, after all, merely geneflow and examining this will further add to our understanding of how the marine environment can be a limiting factor.

This thesis will therefore examine population structure of two ecologically distinct marine predators within the natural laboratory that is the Mediterranean Sea. I

will then take one of those species and compare its genome with that of another, from which it diverged around a million years ago, providing insights in to this process.

1.6.1 Selection of study species

From its initial concept, the examination of the population structure and evolutionary drivers of *Tursiops truncatus* was fundamental to this thesis. This was largely due to the availability of a significant tissue archive, held jointly by Durham University and the University of Lincoln, and the pre-existing expertise in this species in the Molecular Ecology Group at Durham University (see Natoli *et al*., 2005).

However, inclusion of a second species for comparison was a logical expansion of the initial concept to facilitate a broader investigation into evolutionary drivers of marine predators. Substantial consideration was given to selecting an appropriate species for a comparative study. Selecting another cetacean that is equally distributed across the Mediterranean, such as Common Dolphin *Delphinus delphis* or Striped Dolphin *Stenella coeruleoalba*, was briefly considered. Unfortunately, *D. delphis* is now relatively rare within the Mediterranean (Piroddi *et al*., 2011) and acquisition of an appropriate number of samples required for a population genetics study within the timeframe of a doctorate programme was deemed likely unfeasible. By contrast, *S. coeruleoalba* appears commonly within the Mediterranean Sea but with a diet remarkably similar to *T. truncatus* (Öztürk *et al*., 2007; Würtz and Marrale, 1993) and high time investment in socialization (Carlucci *et al*., 2015) it was thought that its genetic population structure is most probably influenced by similar factors and would thus be less than ideal for investigating broader marine predator evolutionary biology.

Seeking greater contrast, large and predatory members of the Elasmobranchii or Osteichthyes were considered. These two taxa contain numerous species that feed at a similar trophic level as identified in *T. truncatus* but perhaps importantly largely lack the social structure present in this species. This absence of social influence allows a *de facto* examination of the importance of this factor for forming population structure in marine predators. Of the Elasmobranchii a number of species could provide potentially useful comparisons, most notably *Isurus oxyrhincus*, *Carcharhinus falciformis* and *Lamna nasus*. However, like with *D. delphis*, encounters with these species within the Mediterranean Sea are rare and sporadic and the risk of being unable to obtain an appropriate number of samples was deemed too high.

The Osteichthyes presented numerous potential comparative species. Most notable are the tunas, represented in the Mediterranean by *Thunnus thynnus*, *Thunnus alalonga*, *Auxis rochei*, *Euthynnus alletteratus*, *Sarda sarda* and *Katsuwonus pelamys*. Of these, the genetic population structure of *Thunnus thynus* has already been the subject of multiple studies owing to its commercial importance (Boustany *et al*., 2008; Carlsson *et al*., 2004; Riccioni *et al*., 2010; Viñas *et al*., 2011) and such studies have revealed strong population structuring within the Mediterranean (albeit with contrasting results). The opportunity to examine this structuring in greater resolution through the application of the ddRADseq methodology (Peterson *et al*., 2012) could be considerably revealing. However, following an offer of existing samples of *Sphyraena viridensis* from the author of Milana *et al*. (2014) it was deemed that this would make an ideal comparative species. As explored in Chapter 3, *S. viridensis* has a suitable geographic distribution for comparison, similar dietary habits to *T. truncatus* and crucially samples are readily attainable through multiple sources.

1.6.2 Aims and hypotheses

This thesis, over the course of three data chapters, will examine and test the following hypotheses:

Hypothesis 1:

H1: Steep marine environmental gradients, such as ocean fronts, can act as a barrier to gene flow in marine predator populations and thus influence population structure.

Hypothesis 2:

H1: Population structure in marine predators is influenced by environmental gradients, either directly or through an alternative mechanism such as influencing the distribution of prey resources.

Hypothesis 3:

H1: Historical speciation, admixture events or demographic changes in marine predators have been influenced by environmental events or changes.

Drivers of genetic population structure for *Tursiops truncatus* in the Mediterranean and eastern Atlantic

2.1 Introduction

This chapter will examine, in detail, the population structure of *Tursiops truncatus* (Montagu, 1821) using Next Generation Sequencing (NGS) technologies. Alongside corresponding analysis of diet and by incorporating environmental factors, this will allow investigation into the drivers of population structure formation in this species; as well as provide insight into the wider evolutionary drivers of the genus. The Mediterranean Sea, as described previously in section 1.4, with its well documented environmental and oceanographic features, provides a suitable context in which to investigate these processes.

2.1.1 Tursiops truncatus - general overview

Tursiops truncatus, or the Common Bottlenose Dolphin, has an almost global distribution, being absent only from polar waters. There is now substantial evidence that, like in Orca *Orcinus orca* (Dahlheim *et al*., 2008; Herman *et al*., 2005), both offshore and coastal ecotypes exist (Hoelzel *et al*., 1998), at least in the western hemisphere. Adults vary in size dependant on geographic location, with warm-water populations reaching 2-3m in length (Sergeant *et al*., 1973) and some temperate populations growing as large as 4m (Avant, 2008). There is sometimes a pronounced sexual dimorphism with males growing larger than females (Shirihai *et al*., 2006) but this is not found in all locations and there is some debate as to whether this can be

described as a defining feature of this species (Hale *et al*., 2000; Hersh *et al*., 1990; Hohn, 1980; Mead and Potter, 1990; Read *et al*., 1993; Sergeant *et al*., 1973). Colouration is varying shades of grey with countershading (paler ventral surface) and with mottled patterns often present. Maximum age of wild *T. truncatus* is unknown but records of individuals attaining 50 years exist (Barros and Wells, 1998). Sexual maturity for both sexes is attained by 14 years (Wells *et al*., 2002).

A typical swimming speed for *T. truncatus* would be up to 11km/h but it is known they can sustain speeds of up to 35 km/h for short periods. *T. truncatus* individuals in the UK have been recorded making movements of more than 150 kilometres over a maximum 48 hour period (E. Cunningham pers. comm. 2018; Pesante *et al*., 2008; Feingold and Evans 2014) making them a highly mobile species and comparable to records of other highly mobile odontocetes (Genov *et al*., 2012). This level of dispersal potential should be kept in mind when considering evidence of gene flow in later discussions.

Figure 2.1: The Bottlenose Dolphin, *Tursiops truncatus*, as photographed offshore from Torretta Granitola, Sicily, during fieldwork for this thesis. © Daniel Moore/IAMC-CNR

2.1.2 Known global genetic structure

Globally, there are currently two recognised species of Bottlenose Dolphin (Committee on Taxonomy, 2018), of which *Tursiops truncatus* is the most widely distributed (Jefferson *et al*., 2015). The other species, designated based on differences in morphology and genetics, is *T. aduncus* (Kemper, 2004; Möller and Beheregaray, 2001; Perrin *et al*., 2007; Wang *et al*., 1999) and is found in coastal regions of the Indo-Pacific. A third species, *T. australis* found in coastal waters of Australia, has been proposed (Charlton *et al*., 2006; Charlton-Robb *et al*., 2011; Möller *et al*., 2008) but is not currently accepted (Committee on Taxonomy, 2018). The Black Sea population of *T. truncatus* is proposed as a subspecies *T. truncatus ponticus* (Viaud-Martinez *et al*., 2008).

T. truncatus has been the subject of a large number of population genetics studies yet a good global understanding remains elusive. Smaller scale studies, looking at regional population structure are much more common (Gaspari *et al*., 2015a; Krützen *et al*., 2004; Martien *et al*., 2012; Sellas *et al*., 2005; Urian *et al*., 2009). However, a few studies have tried to focus on a larger scale. Natoli *et al*. (2004) made use of both mtDNA and microsatellite markers to examine dolphins from seven different regions and found significant differentiation between all regions examined. As in more regional studies there was genetic distinction between coastal and offshore ecotypes but interestingly their data suggests less genetic variation within inshore populations, with the authors suggesting that coastal populations were established by small numbers of the more diverse offshore population. It has been suggested that the niche filled by the coastal ecotype in the western hemisphere is filled by *T. aduncus* in the coastal regions of the Indian and Western Pacific Oceans (Tezanos-Pinto *et al*., 2009). Tezanos-Pinto *et al*. (2009) found that regardless of habitat use, dolphins of the

Pacific differentiated to a much greater degree from Atlantic coastal ecotypes than from Atlantic offshore ecotypes, suggesting greater gene flow among offshore groups and thus supporting the suggestions of Natoli *et al*. (2004). A study of mitochondrial sequences of *T. truncatus* from the oceanic islands of the Azores and Madeira revealed no genetic differentiation to other oceanic areas of the North Atlantic region, further demonstrating the high levels of gene flow in offshore populations (Quérouil *et al*., 2007). Interestingly, the high level of gene flow between the offshore islands of the Azores and Madeira is supported by studies of acoustic features of *T. truncatus* vocalisation, which found these locations shared common acoustic characteristics but were differentiated acoustically from the Mediterranean Sea (Papale *et al*., 2014).

2.1.3 Population structure of Tursiops truncatus in the Mediterranean

The first examination of *T. truncatus* population structure in this region (Natoli *et al*., 2005) remains the most comprehensive, spanning the entire East-West length of the Mediterranean and utilising 74 samples (and 51 samples from other areas such as the Black Sea and eastern North Atlantic), it provides our baseline understanding (Figure 2.2). Natoli *et al.* (2005) examined nine microsatellite loci and the mitochondrial control region and found a major east-west divergence across the Mediterranean with the Siculo-Tunisian front (STF) coinciding geographically with the emergent point of genetic dichotomy, with the authors thus proposing the STF as a barrier to gene flow.

No study since Natoli *et al*. (2005) has been as geographically expansive, concentrating instead on regional areas or individual seas. Gaspari *et al*., (2015a) examined fine-scale population structure within the Adriatic Sea and found that there were high levels of genetic divergence between Adriatic Sea sub-regions as well as

between neighbouring sea regions (Tyrrhenian and Ionian). It has been suggested that coastal populations within the Adriatic have experienced a recent genetic bottleneck with evidence of gene flow from the Ionian Sea and possibly other Mediterranean regions (Galov *et al*., 2011). Post-glacial expansion of offshore populations into coastal areas as sea level rises has been suggested as a probable cause of this gene flow as well as a mechanism for the founding of coastal populations (Gaspari *et al*., 2015b).

Beyond the Mediterranean, it has been shown that *T. truncatus* found in the Black Sea form a distinct and largely isolated population (Moura *et al*., 2013; Natoli *et al*., 2005; Viaud-Martinez *et al*., 2008). In the Macaronesian archipelagos (Azores, Canaries and Madeira), oceanic *T. truncatus* groups form a contiguous population with high levels of gene flow and genetic diversity (Quérouil *et al*., 2007). This contiguous oceanic population is supported by studies that have shown low levels of site fidelity around the archipelagos (Castrillón *et al*., 2011; Tobeña *et al*., 2014; Walton *et al*., 2007) and shared acoustic characteristics of whistle-based communications (Papale *et al*., 2014).

Elucidation of population structure using non-genetic techniques has been attempted. For example, Carnabuci *et al*. (2016) examined social networks of *T. truncatus* in the Pelagos Sanctuary (Notarbartolo-di-Sciara *et al*., 2008) and found that restrictions in network connections coincided with habitat breaks, suggesting a restriction in gene flow too. Stable isotopes from skin of *T. truncatus* stranded on Spanish shores showed individuals from the Balearic Islands exhibited significantly different signatures to those found on the mainland with the suggestion that the deep water of the Balearic Sea may act as a barrier for the species (Borrell *et al*., 2006).

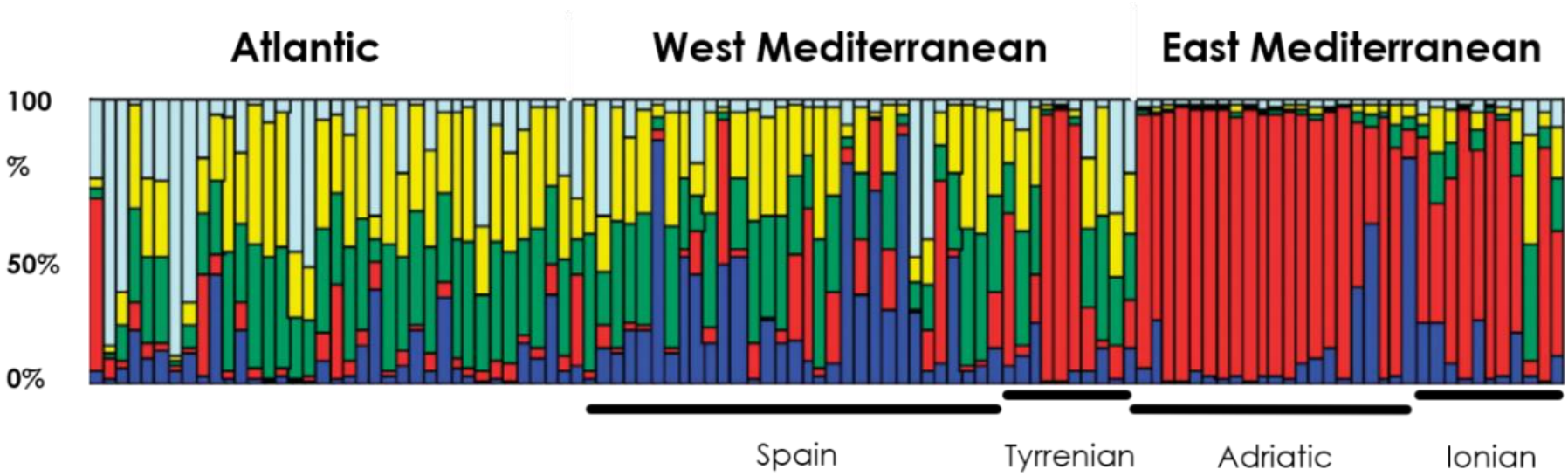

Figure 2.2: Our baseline understanding of genetic population structure of *Tursiops truncatus* in the Mediterranean and North eastern Atlantic. Figure shows estimated proportions of the coefficient of admixture of each individual's genome that originated from population K, for K= 5, created using *STRUCTURE*. Each individual is represented by a vertical column and further geographic location data is given below by the solid black lines. Modified from Natoli *et al.* (2005).

Morphological differences in phenotype may also indicate genetic divergence, as observed between ecotypes (Félix *et al*., 2018; Simões-Lopes *et al*., 2019), and it has been suggested that the reported Levantine nanism in *T. truncatus* may be a product of genetic isolation (Sharir *et al*., 2011).

Following the concurrence of acoustic characteristic data with genetic data found in the Macaronesian *T. truncatus* (Papale *et al*., 2014), it has now been demonstrated that *T. truncatus* found around the Italian island of Sicily also share characterisation of their whistle communications with their Macaronesian counterparts (La Manna *et al*., 2017). It remains to be seen whether this is also concurrent with any genetic association.

2.1.4 Ecotypes

It is well known that *Tursiops truncatus* exhibits differential niche specialisation in the form of two ecotypes: offshore and coastal (Hoelzel *et al*., 1998; Lowther-Thieleking *et al*., 2015; Perrin *et al*., 2011; Rossbach and Herzing, 1999). Within the Mediterranean the Ionian basin has been identified as potentially hosting an offshore ecotype population (Gaspari *et al*., 2015b) and it is thought that the coastal populations found within the Mediterranean were established through a series of founder events by Atlantic offshore ecotype populations (Moura *et al*., 2020).

As the name suggests, the coastal ecotype is strictly found in nearshore waters across the species range. The offshore ecotype, however, is found in pelagic waters but is known to frequent and in some circumstances cohabit in coastal waters with the coastal ecotype (Simões-Lopes *et al*., 2019). It has been suggested that the ecotypes are morphologically distinct; observed differences include skeletal anatomy (Costa *et*

al., 2016; Perrin *et al*., 2011; Toledo, 2013) and the offshore ecotype having darker coloration and a more falcate dorsal fin (Félix *et al*., 2018; Simões-Lopes *et al*., 2019). Interestingly the coastal ecotype is reported as being larger and more robust than its offshore counterpart in the Pacific Ocean (Segura *et al*., 2006), whereas the reverse is true for the north western Atlantic ecotypes where the offshore ecotype is larger (Vollmer and Rosel, 2012). It has been noted that the offshore ecotype has an increased tendency to form large groups of individuals (n>100), possibly linked to foraging, whereas the coastal ecotype is more likely to be observed in smaller pods of twenty individuals or less (Salinas-Zacarias, 2005); though like many ecotype characteristics this may be regionally variable (Hoelzel pers. comm. 2020).

The separation of these two ecotypes is not just a geographical one but also an example of dietary niche specialisation and this can be important when considering its influence on population structure as prey distribution across marine habitats is rarely uniform. Studies utilising Stable Isotope Analysis (SIA) have demonstrated exploitation of different food resources between the ecotypes (Barros *et al*., 2010; Dıaz-Gamboa, 2003; Segura *et al*., 2006) and studies of their teeth show morphological divergence that is consistent with the differential prey targets as suggested by SIA (Perrin *et al*., 2011).

It has now been shown that the two *T. truncatus* ecotypes can also be distinguished genetically (Fruet *et al*., 2017; Hoelzel *et al*., 1998; Torres *et al*., 2003). This level of differentiation, at both phenotypic and genotypic level, has led some to suggest that the two ecotypes could form valid parapatric sub-species (*T. truncatus gephyreus* [coastal ecotype] and *T. truncatus truncatus* [offshore ecotype]) (Costa *et al*., 2016) or even valid species (*T. gephyreus* and *T. truncatus*) (Wickert *et al*., 2016).

The systematic placement of these ecotypes is complex and with widespread geographical variation scientific debate continues (IWC, 2018).

2.1.5 Trophic relationships & feeding

The diet of *Tursiops truncatus* is dominated by fish and cephalopods, an observation made in a variety of populations worldwide (Barros and Wells, 1998; Blanco *et al*., 2001; Gladilina and Gol'din, 2014; Pate and McFee, 2012; Santos *et al*., 2007, 2001). There is evidence from early studies on captive dolphins showing that adult dolphins require between 34 and 67 kcal per kilogram of body weight as a baseline calorific intake each day. This is even higher for subadults at up to 81 kcal per kilogram (Reddy *et al*., 1994). It could be supposed that in the wild such a figure would be substantially higher, to account for active hunting and travel over often considerable distances. However, these values must be treated with caution; it is thought that in some geographical areas, wild larger *T. truncatus* can weigh around 450 kilograms, making an improbable daily energy requirement of 30,150 kcal for an adult. Nevertheless, the dietary energy intake for wild *T. truncatus* in regions of cooler water is likely to be high, something which is reflected in the preponderance of oily, calorie-rich fish often found in their diets (Santos *et al*., 2001). Most prey fish species targeted by *T. truncatus* resident to Europe come from the Gadiformes and include Cod *Gadus* spp., Hake *Merluccius* spp., Saithe/Pollack *Pollachius* spp. and Whiting *Merlangius merlangus* (Blanco *et al*., 2001; González *et al*., 1994; Santos *et al*., 2001). It is also common for *T. truncatus* to target Salmoniformes (Hernandez-Milian *et al*., 2015; Ryan *et al*., 2010).

Aside from the dietary differences between ecotypes already mentioned, there is evidence of variation in diet specialisation between populations, even sympatric ones (Fernández *et al*., 2011a). This arrangement of niche specialisation can allow some coastal marine habitats to have a greater carrying capacity for top predators than would be expected in an equivalent terrestrial setting. Diets are also not temporally stable, at least not at the individual level; stable isotope studies have revealed good evidence for strong ontogenetic diet shifts in *T. truncatus* (Knoff *et al*., 2008) with changes in the nitrogen isotope ($\delta^{15}N$) values in teeth suggested to be a reflection of the shift from mother's milk to prey items during the first year of life.

2.1.6 Diet in Mediterranean Tursiops truncatus

What we know of the diet of *T. truncatus* found within the geographic scope of this study comes mainly from Stomach Contents Analysis (SCA). These studies are fairly few but all indicate a similar diet to that seen for *T. truncatus* in other parts of the world. Blanco *et al.* (2001) examined the stomachs of 16 *T. truncatus* that were stranded around the Spanish coast and found that, as in other ocean areas, larger fish species and to a lesser extent cephalopods were the main prey species. In particular, the European Hake *Merluccius merluccius* was the most abundant prey item found. The authors of this study noted that the composition of prey species indicates that *T. truncatus* in this area are mostly demersal hunters, suggestive of a coastal ecotype.

In a study of a single *T. truncatus* individual that was stranded off the coast of Croatia, *M. merluccius* was also found to be the most abundant prey species present in the stomach, alongside Conger Eel *Conger conger*. Similar to other studies, fish made up 75% of diet composition and the remaining 25% constituted a single

cephalopod species (Miokovi *et al*., 1999). Pedà *et al*. (2015) studied the abundance and composition of cephalopod species found in the stomachs of a number of cetacean species that stranded along the coast of Tuscany, Italy, including 13 *T. truncatus*, and found that of all cetaceans studied *T. truncatus* had the lowest proportion of cephalopods in its diet.

In the Gulf of Cádiz, it has been shown by SCA that *M. merluccius* and *C. conger* are also the dominant prey species of *T. truncatus*. Interestingly however, Stable Isotope Analysis (SIA – see section 2.1.7 for a short primer) in the same study, which gives an indication of 'assimilated diet' over time, suggests that *T. truncatus* may have a diet that is in fact more diverse and from a broader range of fish species not indicated by SCA alone (Giménez *et al*., 2017).

SIA was also employed by Scheinin *et al.* (2014) to examine if *T. truncatus* off the coast of Israel were actively competing with commercial fishing boats for prey species and recorded $\delta^{15}N$ values indicating that Levantine Sea *T. truncatus* were feeding at a lower trophic level than in other areas of the Mediterranean. It is possible that this could be a characteristic of other far eastern Mediterranean *T. truncatus* as it has also been shown by prey scale analysis that for *T. truncatus* feeding in the Greek Gulf of Ambracia the dominant prey sources were small epipelagic planktivorous fishes such as Pilchard *Sardina pilchardus* and Round Sardinella *Sardinella aurita* (Bräger *et al*., 2016).

T. truncatus diets around the Crimean Peninsula in the Black Sea is known from an SCA study of 11 stranded individuals which showed that although their diet was predominantly fish, it was also quite diverse and that species composition was

typically comprised of smaller species than observed in the western Mediterranean and Adriatic (Gladilina and Gol'din, 2014).

2.1.7 Stable Isotope Analysis (SIA): A short primer

There are a number of elements that occur naturally in several stable forms. These forms, called stable isotopes, vary only in the number of neutrons found in their atomic nucleus. For example, the two most common elements used in SIA to answer ecological questions are carbon and nitrogen. Carbon has two stable isotopes: ^{12}C with 6 neutrons and the 6 protons defining it as carbon and ^{13}C which has a nucleus containing 7 neutrons, thus making it slightly heavier than ^{12}C. Similarly, nitrogen also has two stable isotopes: ^{14}N with 7 neutrons and 7 protons and ^{15}N which contains a single extra neutron.

In nearly all cases the heavier isotope of any given element is rarer. In the case of carbon and nitrogen ^{13}C makes up only 1.108% of all carbon atoms found naturally on earth and ^{15}N makes up an even smaller 0.365% of all nitrogen atoms found naturally (Sulzman, 2007). Given that the number of electrons and protons are the same between isotopes this means that chemically or qualitatively they are regarded as the same and will engage in the same chemical reactions. However, the variation in atomic mass means that the behaviour, particularly with regards to reaction rate, will vary between isotopes, thus quantitively they are considered to be different. This means that, following a chemical reaction, the ratio of heavy to light isotopes will likely differ from that found naturally, owing for the increased rate of reaction expressed by the lighter isotope.

The ratio of heavy to light isotopes is always written in a standard notation expressed in relation to a globally accepted standard for each element. The notation is:

$$\delta X = \frac{R_{sample} - R_{std}}{R_{std}} \times 1000$$

Where δ is the isotopic notation, X is the heavier isotope of the element (^{13}C or ^{15}N in this thesis) and R is the ratio between heavy and light isotopes. The notation is always expressed in parts per thousand (‰). The standards for $\delta^{13}C$ and $\delta^{15}N$ are Vienna Peedee Belemnite and atmospheric nitrogen respectively. When isotopic ratios are reported in the standard notation the sign of delta (δ) is important; A positive δ indicates that the sample being measured has an increased percentage of the heavier isotope relative to the standard, whereas a negative δ indicates that the sample has less of the heavier isotope than the standard.

As highlighted, the quantitative difference between isotopes results in a difference between the ratio of isotopes before and after a chemical reaction and this is termed fractionation. Where fractionation results in a greater proportion of the heavier isotope in the reaction products than the reactants then this is termed enrichment. Where the products have less of the heavier isotope than the reactants then this is termed depletion. When a reaction tends to favour one isotope over another this is sometimes referred to as discrimination.

To put these terms in to a simple context we can consider evaporation of water from the ocean's surface and its effect on stable isotopes of oxygen. The water in the ocean contains a mix of oxygen isotopes (^{16}O, ^{17}O and ^{18}O in a ratio of 99.759:0.037:0.204). When the water evaporates it takes more energy to convert the water molecules containing ^{18}O into water vapour than it does those molecules

containing only ^{16}O due to the increased mass (from the extra neutrons). This results in a fractionation whereby the resultant water vapour is depleted in ^{18}O (in this case $\delta^{18}O_{water\ vapour}$ = -13.0‰) because the process of evaporation discriminates against the heavier isotope.

This discrimination in reactions and its resultant fractionation can make stable isotopes a very useful tool in ecological research. As organisms consume organic matter from others the physiological processes (such as enzymatic reactions) that occur in the process of assimilating required molecules or discarding ones that are not needed results in fractionation. For $\delta^{15}N$ and $\delta^{13}C$ this typically results in enrichment at each trophic level (Figure 2.2), being especially true for $\delta^{15}N$. This means that when we examine the tissues from two populations of the same predator species found in differing geographic locations and notice a difference in $\delta^{15}N$ it is likely to indicate that they are utilising differing prey resources (there are caveats however – see below for further explanation of these). Due to trophic enrichment the population with the greater enrichment of ^{15}N is thus feeding at a higher trophic level.

Consideration must always be taken for geographic variability in enrichment at the base of food chains when making comparisons between geographically separated predator populations as such enrichments will cascade up each level (Jennings *et al*., 1997). This is especially true in marine environments where nearshore habitats are typically depleted in ^{15}N due to input from ^{15}N depleted terrestrial runoff. This geographic differential is exacerbated by pelagic habitats being enriched in ^{15}N due to accumulation of ^{15}N enriched NO_3^-. There are also significant differences in $\delta^{13}C$ between coastal and pelagic habitats owing to differing levels of dissolved CO_2, as a result of differences in temperature and phytoplankton growth rates (Hobson, 1993; Michener and Kaufman, 2007). This geographic variation in $\delta^{13}C$ can be useful

in differentiating between the two *Tursiops truncatus* ecotypes, with offshore ecotypes usually being slightly depleted for ^{13}C (Barros *et al*., 2010), and for estimating marine animal movements by utilising SST as a proxy for phytoplankton induced $\delta^{13}C$ variation (MacKenzie *et al*., 2011) but equally it does introduce problems when interpreting trophic level of populations spread over a wide geographic area. This can be overcome by reliable estimation of baseline input of isotopic ratios into any given system, often referred to as the fixed isoscape approach, usually derived from in situ sampling of lower trophic level species (bivalve molluscs make good target species owing to their sessile lifestyle and that their tissues act as a *de facto* average of water column isotopic ratios over time – see Barnes *et al*., 2009). Owing to the expensive and time-consuming nature of developing fixed isoscapes there remains limited global coverage and none currently exist for the geographical area covered by this thesis. However, this does not prevent the use of SIA over broad geographic areas for trophic level assessments, indeed many studies have taken this approach (Ryan *et al*., 2013; Santos *et al*., 2013). Such studies assume a constant baseline of environmental isotopic ratios across the study area which can't be validated. As a result, substantial extra caution must be exercised in the interpretation of these data, particularly when comparing coastal to offshore areas due to the processes described above.

A cautious approach is also necessary where SIA studies make use of multiple tissue types and/or multiple storage methods (Lesage *et al*., 2010; Sarakinos *et al*., 2002; Willis *et al*., 2013). Both factors can influence isotopic ratio and an understanding of this can aid more accurate interpretation of data. Unfortunately, tissue variation can be species specific without a predictable relationship across taxa (Hussey *et al*., 2012). As such, validation studies, which are currently lacking for many species, are needed to support future SIA work. Until such time as these studies are

available efforts should be taken to 1) avoid cross-tissue comparisons, 2) where sample sizes allow apply discrimination factors to normalise data across tissues and 3) in all cases apply extra caution to the interpretation of these data.

2.1.8 Behaviour and social structure

Tursiops truncatus are a highly communal predator with complex social structures (Blasi and Boitani, 2014; Miller *et al*., 2010; Rogers *et al*., 2004). It is likely that their social structures and behaviours play a significant role in gene flow patterns and formation of population structure, as have been demonstrated in other cetacean species (Pilot *et al*., 2010).

There are differences between the *T. truncatus* ecotypes, as previously mentioned, with offshore ecotype groups tending to form larger social groups (Salinas-Zacarias, 2005) but social interaction, it would seem, is universally important no matter the scale. It is even reported that individuals that are isolated from their populations will seek other forms of social interaction, even with members of other species (Lockyer, 1978; Wilke *et al*., 2005).

Society in *T. truncatus* is based on a fission-fusion dynamic structure rather than temporally stable groups (Lewis *et al*., 2011). Studies of social networks in *T. aduncus* show large variation in sociability with some individuals maintaining interactions with hundreds of other individuals over their lifetime whilst some more reclusive dolphins may only maintain connections with a few tens of others (Gibson and Mann, 2008). Although there seems to be evidence for individuals to preferentially associate in set cliques with known individuals, the lack of community boundary and

the high likelihood of stranger interaction has potential implications for gene flow between populations.

The strongest social bonds in Bottlenose Dolphin societies tend to form between mother and offspring. Observations of *T. aduncus* show that during weaning, which can last anywhere between three and seven years, the offspring will rarely leave its mother's side (Mann *et al*., 2000). Although not true for all populations, females tend to remain in their natal groups following weaning whereas males often form single-sex juvenile groups (Tsai and Mann, 2013). These juvenile male groups are often quite mobile and the high levels of interaction between members can lead to life-long alliances. These life-long alliances (called first-order alliances) usually form between groups of two to three individuals who are then observed to work together to gain access to females. On occasion, multiple first-order alliances can form larger temporary groups called second-order alliances to serve the same purpose (Connor *et al*., 2011). Although there is likely regional variation (see Papale *et al*., (2017)) many of the aforementioned social characteristics are likely common across *Tursiops* sp.

2.1.9 Genetic approaches to assessing population structure of T. truncatus

All previous studies of genetic population structure of *T. truncatus* in the Mediterranean have been conducted using traditional Sanger sequencing (Sanger and Coulson, 1975) on 'first generation' sequencing machines. Sanger sequencing is generally regarded as having a lower discovery power, lower sensitivity and poor cost effectiveness for high numbers of DNA targets when compared to Next Generation Sequencing (NGS). When using a comparable number of Single Nucleotide Polymorphisms (SNPs) and microsatellite markers it has been shown that SNPs are

still able to elucidate population structure (Coates *et al*., 2009), however the fact that NGS allows for processing of many thousands of SNPs means that the resolution of NGS derived SNP datasets is far higher. Furthermore, microsatellite markers have been shown to give inflated F_{ST} values when estimating genetic differentiation between populations in controlled studies alongside SNP markers due to a number of possible causes (limited number of microsatellite markers used, marker ascertainment bias, as well as the high variance in microsatellite-derived estimates) (Fischer *et al*., 2017).

Mitochondrial DNA sequences have been praised for their simplicity of use and ease by which studies can be replicated between institutions but it is observed that they can have limited ability to resolve population structure where differentiation is low or gene flow is high (Morin *et al*., 2004). Whilst mitochondrial DNA might always be considered useful as a haploid matriline marker, thus providing some demographic inference, the ability of SNPs to resolve subtle differentiation between populations mean that they increasingly out-perform previously used markers for population structure studies.

This study will utilise NGS, and specifically the ddRADseq methodology (Peterson *et al*., 2012), to develop a SNP dataset to overcome the methodological limitations of past studies of population structure in Mediterranean *Tursiops truncatus*, greatly improving our genetic resolution and capacity to understand drivers of structure formation.

2.1.10 Aims and hypotheses

This chapter will examine and test the following hypotheses:

Hypothesis 1:

H1: Population structure in Mediterranean *Tursiops truncatus* is significantly influenced by the presence of ocean fronts.

Hypothesis 2:

H1: Differential diets and/or prey specialisation between populations of *Tursiops truncatus* in the Mediterranean can restrict inter-population geneflow and is thus reflected in the observable population structure.

Hypothesis 3:

H1: Observable population structure of *Tursiops truncatus* in the Mediterranean is correlated with one or multiple environmental variables, which may in turn be a driver of such structure.

2.2 Methodology

2.2.1 Sample collection

A large proportion of samples used in this study (n=131 out of a total of 176 samples) are derived from an archive held jointly between the Department of Biosciences, University of Durham and the School of Life Sciences, University of Lincoln. This archive has been utilised by numerous previous studies (Gaspari *et al*., 2015b, 2015a; Moura *et al*., 2013; Natoli *et al*., 2005, 2004) and consists of DNA extractions as well as tissue samples of various origin (Skin, Blubber, Muscle etc.). Individual dolphin samples within the archive may comprise just extracted DNA, just tissue or both tissue and extracted DNA. The archive has been built up by multiple researchers over a long time period and is derived from samples donated from many collaborative partners across the Mediterranean and eastern Atlantic region. As may be expected from an archive established in this manner, available metadata for each sample is sporadic and varies from little to comprehensive, dependent on the collecting organisation. All samples contained within the archive were collected from either stranded animals or through biopsy sampling. In addition to archived samples, additional samples (n=8) for this study were collected during a biopsy sampling campaign in Sicily, Italy during September 2017, owing to a lack of archive samples in this geographic region. Additional samples were kindly donated by Dr Mónica Silva of the Marine and Environmental Sciences Centre, Azores (n=29, all of which were collected by biopsy sampling) and Professor Juan Antonio Raga of the University of Valencia (n=8, all of which come from stranded animals). Metadata, including sample origin, for each sample used in this study is provided in Appendix IV.

2.2.2 Strandings

Strandings of *Tursiops truncatus* are relatively rare compared to other small cetacean species but can occur for a number of reasons (Dunn *et al*., 2002; Lahvis *et al*., 1995; Lipscomb *et al*., 1996, 1994). Stranded animals may have drifted from their place of death so location should be treated as regional rather than absolute. This movement, principally by tides or currents, may contribute to noise in population structure due to population mis-assignment. Furthermore, degradation of samples from stranded animals means there may be a selective bias for biopsied animals due to the likely increased DNA quality after extraction.

At least 38 samples used in this study were derived from stranded animals (in reality this figure may be higher but a proportion of samples stored in the archive lack the comprehensive metadata for their source to be ascertained). A number of different organisations contributed samples from stranded animals (see Appendix IV for details). Stranded samples were collected between 1991 and 2011. Procedures for collecting and storing tissue samples from stranded animals can vary hugely between organisations and as such the tissues used in this study included skin, muscle, blubber, heart, kidney, lung and liver. The storage of such samples also varied and included simple freezing, freeze-drying and storage in Dimethyl Sulphoxide (DMSO) or ethanol. This is of note as the variation in tissue type and storage method may have an influence on certain analyses, particularly stable isotope analysis, which is discussed later.

2.2.3 Biopsy sampling

At least 37 samples from the archive were retrieved through biopsy sampling. Further biopsy sampling was conducted off the coast of Torretta Granitola, Southern Sicily, Italy during the period 4th to 19th September 2017 (Figure 2.3) in collaboration with the Institute for Coastal Marine Environment of the National Research Council (IAMC-CNR) and Tilen Genov of Morigenos. Biopsy sampling was conducted using a Petron Stealth Wood Stock Crossbow with a 150lb draw-eight and detachable iron sights (Figure 2.4A). Custom biopsy bolts were obtained from Ceta-Dart V/Finn Larsen and consisted of Easton ACC 3-71 shafts (with vanes and nock and a M8 thread) and detachable 3 barbed 25mm biopsy tips (with an M8 thread) (Figure 2.4B-D). All biopsy tips were sterilised via 100% ethanol and flame prior to use and wrapped in foil to maintain sterile conditions until required for use. Biopsy sampling protocol largely followed the Northeast Fisheries Science Centre Cetacean Biopsy Training Manual (Wenzel *et al*., 2010).

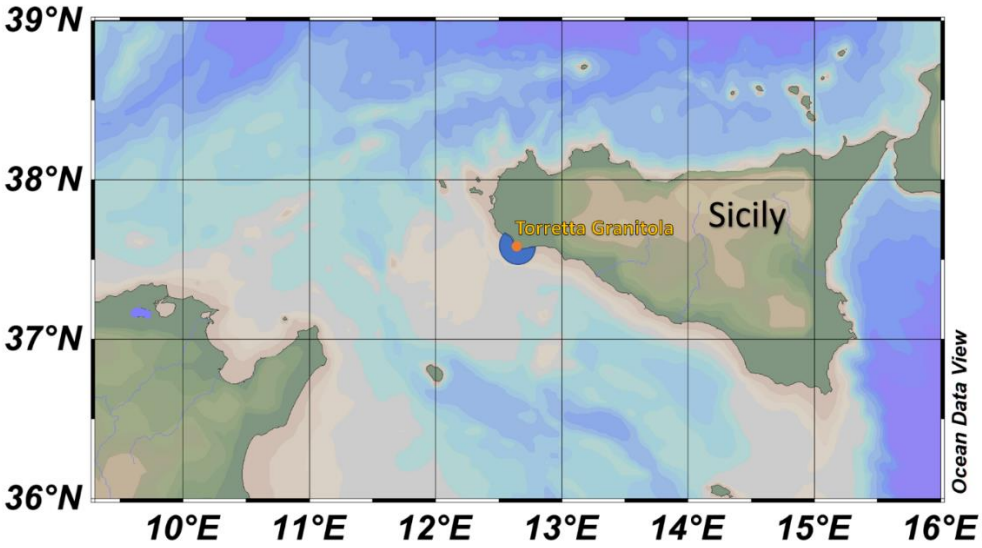

Figure 2.3: Sampling location for Sicily biopsy campaign during September 2017. The town of Torretta Granitola is marked by the solid orange circle. The approximate area of sampling is indicated by the dark blue circular area surrounding Torretta Granitola.

Figure 2.4: Equipment used for the biopsy campaign in Torretta Granitola, Italy. Showing the Petron Stealth Wood Stock Crossbow with a 150lb draw-eight and detachable iron sights (A), the internal barbs of the detachable 25mm biopsy tips (B), the Ceta-Dart V/Finn Larsen custom biopsy bolts with Easton ACC 3-71 shafts (C) and a successful hit showing tissue retained within the biopsy tip (D). (Credits: A-C © Daniel Moore, D © Emily Cunningham.)

It is known that *T. truncatus* in this area commonly feed opportunistically from pelagic trawl vessels (E. Papale, pers. comm. 2017; Alessi *et al*., 2018). In the field it was found that approximately 1 in every 10 fishing vessels were accompanied by *T. truncatus* groups and thus this was an efficient way to locate target animals. During this fieldwork dolphins were encountered away from fishing vessels on only two occasions and in both instances appeared to be mother-calf pairs.

Groups containing mother-calf pairs were deemed unsuitable for biopsy sampling, following best practice guidelines. For other groups, photo-ID was conducted to establish the identity (whether known or unknown) of each target individual and thus ensure avoidance of re-sampling. Photos were compared in real time to a reference catalogue held by IAMC-CNR staff. Acoustic recordings of 2-5

minutes were made immediately before and after each biopsy attempt to assess the impact of sampling on dolphin behaviour.

Dolphins were only targeted when moving parallel to or away from the vessel. Due to the low power of the crossbow it was deemed safe to target bow riding dolphins with minimal risk of injury (Noren and Mocklin, 2012). Biopsy bolts were retrieved from the water using a landing net. The biopsy tip was then removed to retrieve the biopsy sample (Figure 2.4D) which was scored using a sterile scalpel to aid rapid preservation. Samples were stored in salt saturated DMSO in 2ml tubes and kept on ice until return to land whereby they were stored frozen for transportation back to Durham University. Final sample geographic distributions (archived and new biopsy) are shown in Figure 2.5.

Biopsy sampling was conducted under permit granted by *Ministero dell'Ambiente e della Tutela del Territorio e del Mare* (No 33969 – See Appendix VIII). All *T. truncatus* samples were transported to Durham under import authorisation from DEFRA (IMP/GEN/2014/06 33969 – See Appendix VIII) as permitted by the trade in animals and related products regulations 2011.

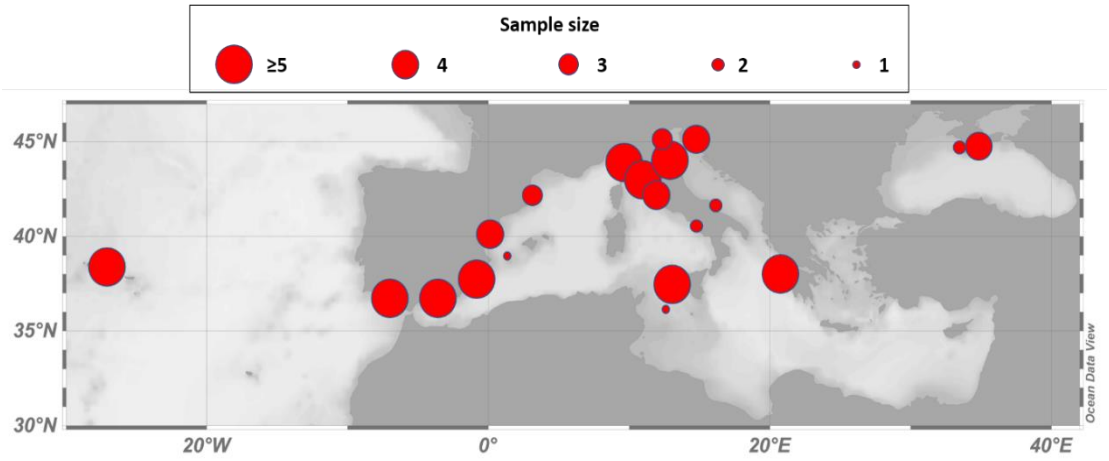

Figure 2.5: Geographic distribution of *Tursiops truncatus* samples used in this study.

2.2.4 DNA extraction

A standard phenol-chloroform extraction protocol was used to isolate DNA, largely following Hoelzel (1998). 45µl of proteinase-K was added to diced tissue samples, which were then digested overnight at 37°C to ensure maximum DNA yield. Additionally, the final aqueous phase, to which was added 2x volume of 100% ethanol and 2% of 2x volume of 3 molar sodium acetate, was stored at -20°C overnight to ensure complete precipitation. The final DNA pellet was then dried in a centrifugal evaporator under vacuum conditions. Drying typically lasted 1-2 hours. The DNA pellet was then resuspended in 50µl TE buffer (10 mM Tris pH 8.0, 1mM EDTA pH 8.0).

Concentration of DNA extractions was evaluated using a Qubit 2.0 fluorometer (Invitrogen). Typically, at least two independent readings would be made of each extraction to ensure confidence of the measurement. DNA extraction quality was assessed using a Nanodrop spectrophotometer (Thermo Scientific).

2.2.5 Library preparation

Genetic libraries for sequencing were developed largely following Peterson *et al*. (2012) (Figure 2.6) with a few modifications. For a complete desktop protocol see Appendix I. 500ng of DNA was subject to restriction enzyme digest using enzymes MspI and HindIII (New England Biolabs) at 37°C overnight in a 50µl reaction. Following successful digestion, samples were ligated with P1 and P2 adapters. Each individual within a given pool was ligated with a unique P1 adapter (or barcode) to facilitate a multiplex approach. Adapters were ligated by Polymerase Chain Reaction (PCR) with an adjusted volume of enzyme digested DNA (based on visual inspection

of electrophoresis gel – to aid sample balancing). Ligated samples were once again run on an electrophoresis gel and visual assessment of sample brightness was used to aid balanced pooling. Pools were cleaned using calibrated streptavidin coated SpeedBeads (Sera-Mag). Concentration of cleaned DNA pools was evaluated using a Qubit 2.0 fluorometer (Invitrogen).

DNA fragments were size selected to a desired length of 325-475bp on a Pippin Prep (Sage Science). Size-selected pools were then amplified and unique pool indices added via PCR. Amplified PCR reactions were then pooled by pool and concentration was evaluated using a Qubit 2.0 fluorometer (Invitrogen). Pools were then cleaned again using calibrated streptavidin coated SpeedBeads (Sera-Mag).

Pool fragment size and contamination was checked using a 2200 TapeStation (Agilent Technologies). Pool concentration was quantified using qPCR and a commercial quantification kit (Kapa Biosystems). For qPCR, dilutions of each pool were made (1 in 1000 and 1 in 5000) to ensure values fell within range of qPCR standards (Kapa Biosystems DNA standards 1-6). Pools were then standardized and combined in equal molarity to form a final library of 10nM concentration.

Figure 2.6: A schematic of the double digest Restriction Associated DNA sequencing technique adapted from Peterson *et al.* (2012). Two enzymes are used to cut genomic DNA and then precise size-selection steps select only those fragments close to the target fragment length, excluding regions either very close (a) or very far (b) from restriction enzyme sites. Multiple sequence reads provide depth to each SNP site.

2.2.6 Sequencing

Final libraries (n = 3) were sequenced on three lanes of an Illumina HiSeq 2500 (100bp paired end reads) at the DBS Genomics facility at Durham University. See the following for further detail:

https://www.dur.ac.uk/biosciences/services/dna/dnasequencing/.

The sequencing of each library was conducted on three separate occasions: Library 1 in December 2015, Library 2 in September 2016 and Library 3 in February 2018.

2.2.7 Stable Isotope Analysis

All tissue samples were kept frozen at -20°C prior to preparation for Stable Isotope Analysis (SIA). Available tissue samples were derived from multiple sources (pre-existing archive and fresh biopsies) and tissue types, including skin, blubber, muscle and kidney (See Appendix III for details). Due to a concern for bias in $\delta^{13}C$ values originating from tissues containing large quantities of naturally occurring lipids, it was determined that all samples would be subject to a lipid extraction protocol prior to analysis.

Samples to be analysed in bulk were defrosted and a small subsample, approximately 0.5cm^3 in size, was transferred to a clean prelabelled 1.5ml Eppendorf where it was finely diced using bow scissors. 1000µl of deionized H_2O was then added to the Eppendorf which was then placed in a foam float in an ultrasound bath and sonicated for 15 minutes. Following this the Eppendorf was centrifuged at 3000rpm for 10 minutes before the H_2O was carefully removed and the Eppendorf was placed open in a drying oven heated to 45°C until the sample was fully desiccated.

All other samples were subject to lipid extraction as follows. Samples were defrosted and a small subsample, again approximately 0.5cm^3 in size, was transferred to a clean prelabelled 1.5ml Eppendorf. Then, 1000µl of 3:1 dichloromethane:methanol was added to the Eppendorf before it was placed in a foam float in and ultrasound bath and sonicated for 15 minutes. Following this the Eppendorf was centrifuged at 3000rpm for ten minutes before the dichloromethane:methanol mix was carefully removed and retained in a new 15ml tube for potential future analysis of the lipid extract. These steps were then repeated twice more so that the sample had been sonicated in a fresh 1000µl of dichloromethane:methanol mix three times. Upon removal of the final mix the sample was placed in a drying oven heated to 45°C until the sample was fully desiccated.

Once fully desiccated all samples are ground by hand to produce a fine powder. 0.3-0.5mg of sample was then loaded into tin capsules for stable isotope analysis. A desktop protocol can be found in Appendix II.

2.2.8 Mass spectrometry

Analysis of the samples for carbon and nitrogen isotopes was performed at the Stable Isotope & Biogeochemistry Laboratory (SIBL), Durham University using an ECS 4010 Elemental Analyzer (Costech) connected to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific). Correction of carbon isotope ratios for ^{17}O contribution are reported in standard delta (δ) notation in per mil (‰) relative to Vienna Pee Dee Belemnite (VPDB). Isotopic accuracy was monitored through routine analyses of in-house standards, which were stringently calibrated against international standards (e.g., USGS 40, USGS 24, IAEA 600, IAEA CH3, IAEA CH7, IAEA N1, IAEA N2): giving a total linear range in δ^{13}C between –46 ‰ and +3 ‰, and between –4.5 ‰ and +20.4 ‰ for

$\delta^{15}N$. $\delta^{13}C$ and $\delta^{15}N$ analytical uncertainty was typically ±0.1 ‰ or better for replicate analyses of the international standards and <0.2 ‰ for replicate sample analysis.

2.2.9 Analyses

Sequenced library data were deposited on the Hamilton Cluster at Durham University. The Hamilton Cluster is a Linux based cluster that provides a High-Performance Computing service to researchers at this institution. Raw sequencing reads were demultiplexed and quality filtered using the *process radtags* subprogram in *Stacks* v1.35 (Catchen *et al*., 2013). *Process radtags* flags were defined as *-q 10 -t 92 -r –renz_1 msp1 –renz_2 hindIII -E phred33*. These flags ensure a minimum Phred score of 10 (or 90% probability of correct position) and that any reads with a nucleotide position of <10 were removed. A Phred score of 10 is standard in many SNP studies (Scaglione *et al*., 2012; Wagner *et al*., 2013). All reads were trimmed to 92 nucleotides to limit sequencing errors that may be present in read tails.

The *Tursiops truncatus* reference genome Tur_tru_Illumina_hap_v1 (GenBank Accession GCA_003314715.1) was downloaded from NCBI directly to the Hamilton Cluster. Reference indexes were created using the *bowtie2-build* command within *Bowtie2* v2.2.5 (Langmead and Salzberg, 2012). *Bowtie2* v2.2.5 was then used to align all sequence reads to our reference genome using default settings. SNP detection was then completed using the *Stacks* v1.35 (Catchen *et al*., 2013) *ref_map.pl* pipeline with flags set as *-m 3 -n 2*. These flag settings require a minimum of 3x coverage when reporting a stack in the *pstacks* programme and a mismatch of 2 loci when building the catalogue. The population map for *ref_map.pl* was user defined and populations were ascribed according to geographic sampling location (Figure 2.7)

with inference from Natoli *et al*. (2005) to determine population boundaries (see Appendix IV for ascribed sample populations). Any samples with less than 900,000 reads were not included in the population map and thus excluded from any further analysis (Figure 2.8). An initial GenePop file was created using the *populations* subprogram of *Stacks* v1.35. This file was then examined using *Microsoft Excel* to look for those samples with more than 30% missing data, which were also removed from further analysis.

Initial identification of loci putatively under selection was carried out using the software package *Lositan* (Antao *et al*., 2008). *Lositan* was run with the following parameters: 50,000 simulations, a confidence interval rate of 0.95, a false discovery rate of 0.05, Infinite Alleles mutation model and a subsample size of 30. The *Lositan* output table was used to determine outlier loci with those loci falling outside of outlier thresholds being selected and forming an outlier whitelist for input into *populations*. Loci that fell within the outlier thresholds were determined as neutral and formed a neutral whitelist for input into *populations*. Files were edited for cross-compatibility using a combination of *Notepad++* and the in-built Linux editor *Nano*. *Lositan* outlier selection was visualised in *R* using the package *ggplot*. Population structure analyses were implemented on both those loci identified as under positive selection and those determined as neutral to allow for inference on the effects of environmental adaptation on structure.

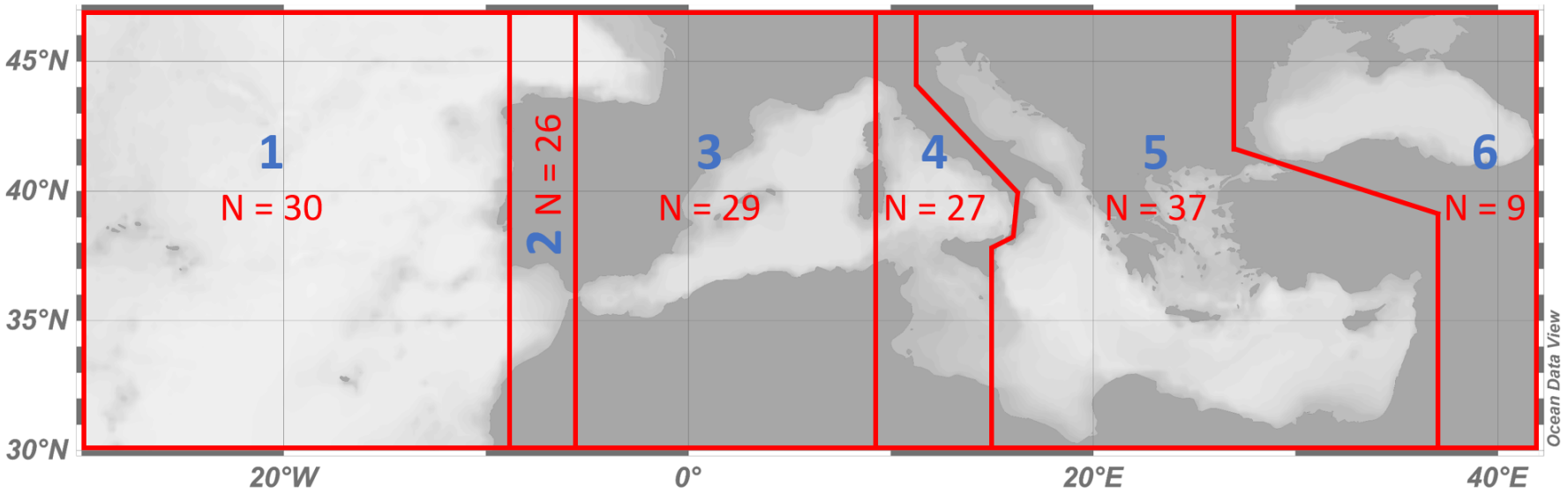

Figure 2.7: Schematic of population map as inputted in to the *Stacks* v1.35 *ref_map.pl* pipeline based on geographic location and Natoli *et al.* (2005).

Population structure analyses were conducted (with separate assessment for neutral and outlier loci, as determined by *Lositan*) using Principal Component Analysis (PCA) (Jolliffe, 2011), Discriminant Analysis of Principal Components (DAPC) (Jombart *et al*., 2010), Additionally, population structure was also assessed using *Admixture* (Alexander *et al*., 2009), as well as by sNMF implemented using the Landscape and Ecological Studies (*LEA*) package (Frichot and Francois, 2015) and a method of ancestral probability based on Minor Allele Frequency (MAF) using an in-house developed *R* package called *SambaR* (de Jong *et al*. unpubl.). All of the aforementioned analyses were implemented in *R* version 3.6.1 (R Core Team, 2019). Analysis of contemporary migration rates (gene flow) was calculated using *BayesAss3-SNPs* (Mussmann *et al*., 2019) and visualised in *R* using *SambaR*. Identification of the strongest restrictions in gene flow between populations was calculated using *Barrier* v2.2 (Manni *et al*., 2004).

Analysis of genetic diversity (genome wide heterozygosity, Minor Allele Frequencies (MAF) calculations etc.) were conducted using *SambaR*. Analyses of genetic differentiation between populations (Nei's genetic D, Pairwise F_{ST} etc.) were conducted using *Arlequin* (Excoffier and Lischer, 2010) and the *R* package *StAMPP* (Pembleton *et al*., 2013).

Investigations of the influence of environment and feeding on population structure was conducted using *GESTE* 2.0 (Foll and Gaggiotti, 2006), Mantel Tests and RDA analysis were conducted within the *R* package *Vegan* (Oksanen *et al*., 2010). Implementation of a Latent Factor Mixed Model (LFMM) analysis was conducted using the *R* package *LEA* (Frichot and Francois, 2015).

Stable isotope data were manipulated in *Microsoft Excel* and isoscape plots were produced using *Ocean Data View* (Schlitzer, 2018).

Additional details pertaining to each analysis, where appropriate, are described below. Principal Component Analysis (PCA) (Jolliffe, 2011) was conducted in *R* using the package Adegenet (Jombart, 2008) and the function *glPca*. A barplot of eigenvalues created from an initial PCA was used to assess the number of eigenvectors (principal components) to retain. The final number of principal components retained was based on the point that the cumulative variance explained by all previous principal components reached a point of diminishing returns. PCA results were visualised using the *R* package *ggplot2* (Wickham, 2016).

Discriminant Analysis of Principal Components (DAPC) (Jombart *et al*., 2010) was conducted in *R* using the package *Adegenet* (Jombart, 2008). Clusters were found using the function *find.clusters* with the maximum clusters (*max.n.clust*) set above the maximum hypothesised number of populations under study (n=6). 100% of the total variance expressed comes from the retained axes of the PCA analysis. DAPC was run with 1,000,000 iterations of each run of K-means algorithm and 1000 randomly chosen centroids.

Estimated admixture coefficients were produced using the function *snmf* in the *R* package *LEA* (Frichot and Francois, 2015). Coefficients were estimated for all values of K from 2-8 and the snmf regularization parameter (alpha) was set at 100. When estimating contemporary migration rates (gene flow) using *BayesAss3-SNPs* (Mussmann *et al*., 2019) the analysis was run with 1,000,000 iterations and a burn-in of 100,000 iterations. Identification of the strongest restriction to gene flow was conducted using *Barrier* v2.2 (Manni *et al*., 2004) and implemented in the *R* package

Adegenet. Input geofiles contained latitude and longitude data in the form of decimal degrees and required data 'jittering' using the function *jitter* to resolve replicate values and from this a Delauney triangulation map was created. Distances were computed from a Nei's genetic D matrix. *Barrier* then applies a Monmonier's maximum distance algorithm to calculate where difference between pairs of populations is greatest. *Barrier* Delauney triangulation maps were then projected onto an *Ocean Data View* created geographical map using *Microsoft PowerPoint*.

To compute pairwise F_{ST} values, calculations were performed in *Arlequin* version 3.5.2.2 (Excoffier and Lischer, 2010), with 100 permutations and a significance level set at 0.05. Calculations were performed with all loci and on separated neutral and outlier loci sets. Nei's genetic distances (Nei, 1972) between populations was calculated using the function *stamppNeisD* in the *R* package *StAMPP*. An in-house built function of *SambaR* was utilised to calculate Weir & Cockerham's F_{ST} (Weir and Cockerham, 1984) values. Both Nei's genetic distance and Weir & Cockerham F_{ST} values were calculated using all loci. Nucleotide diversity, Minor Allele Frequencies, Allele Frequency Spectra and Watterson's theta (θ_W) were calculated using *SambaR*.

Only a limited number of *T. truncatus* samples had tissue suitable for stable isotope analysis (n=79), many were only archived DNA extractions and thus unsuitable. Therefore, in order to maximise use of genetic data for incorporation in to environmental analysis isoscape plots were produced using *Ocean Data View* (Schlitzer, 2018). Isotopic values were projected with DIVA gridding and an x-scale and y-scale length of 60 and 123 respectively. Statistical tests of variability in isotope values between geographic areas were conducted in *Minitab* v14.

Mantel tests were performed to test correlation between genetic distance matrices (Nei's D, calculated via *StAMPP* in *R*) and distance matrices of environmental variables (SST, Salinity, Chlorophyll A, geographical distance and stable isotope values for $\delta^{13}C$ and $\delta^{15}N$ – whilst depth is likely to be ecologically important it varies greatly at even small geographical scales within the Mediterranean and could not therefore be effectively included in this analysis). Tests were performed with localised populations, on outlier loci only using the package *Vegan* in *R* and set with 999 permutations and a Pearson model.

Generalised Linear Models (GLMs), used to test relatedness of localised population F_{ST} values to environmental factors (SST, Salinity, Chlorophyll A and stable isotope values for $\delta^{13}C$ and $\delta^{15}N$), were attempted through *GESTE* version 2 (Genetic Structure inference based on genetic and Environmental data (Foll and Gaggiotti, 2006)). Calculations were run with 250,000 iterations, a burn-in of 50,000 and a thinning interval of 20.

Redundancy Analysis (RDA) was conducted using the packages *Vegan* and *psych* in *R*. RDAs were ran with both neutral and outlier loci with SST, salinity, chlorophyll A, $\delta^{13}C$ and $\delta^{15}N$ as environmental input variables.

To estimate historic demography for local populations the Site Frequency Spectrum (SFS) was calculated using *ANGSD* (Korneliussen *et al*., 2014). This was then used in the Java implemented *Stairway_plot* function to derive the visual output based on a bash script blueprint input file. Mutation rate and generation time were both derived from available literature with mutation rate set at $1.5x10^{-8}$ (Moura *et al*., 2014) and generation time at 21.5 years (Taylor *et al*., 2007).

To detect SNPs which showed strong association with environmental factors Latent Factor Mixed Models (LFMM) were utilised using the *lfmm* function in *R* package *LEA* (Frichot and Francois, 2015). All loci were inputted in to the model and K was set at K=6 (based on earlier snfm analysis). For the input of environmental factors, a single environmental variable was derived from PC1 of a PCA of SST, salinity and chlorophyll A for localised population geographical locations. A burn-in of 5,000 iterations was used in the LFMM followed by 10,000 Markov-Chain Monte Carlo (MCMC) iterations, all of which were replicated five times. Z-scores for each SNP were combined from each of the five replicates, and false discovery rates were then evaluated using adjusted p-values. P-values were adjusted following Benjamini and Hochberg (1995). All loci with an adjusted p-value of less than 0.05 were retained as outliers showing strong association with environmental factors.

2.3 Results

A total of 176 *T. truncatus* individuals were sequenced for this study. 10 samples were removed from further study due to low number of reads (<900,000) and 9 further samples were removed due to high levels of missing data (>30% missing data) (see Figure 2.8). Final p parameters in the *Stacks* v1.35 populations program produced 5104 loci from 166 individuals. Optimum parameters for running populations were found by trialling multiple iterations.

Outlier detection, implemented in *Lositan* (Figure 2.9), discovered 253 loci putatively under positive selection. Thresholds also indicated 3476 and 1375 loci putatively neutral and under balancing selection respectively.

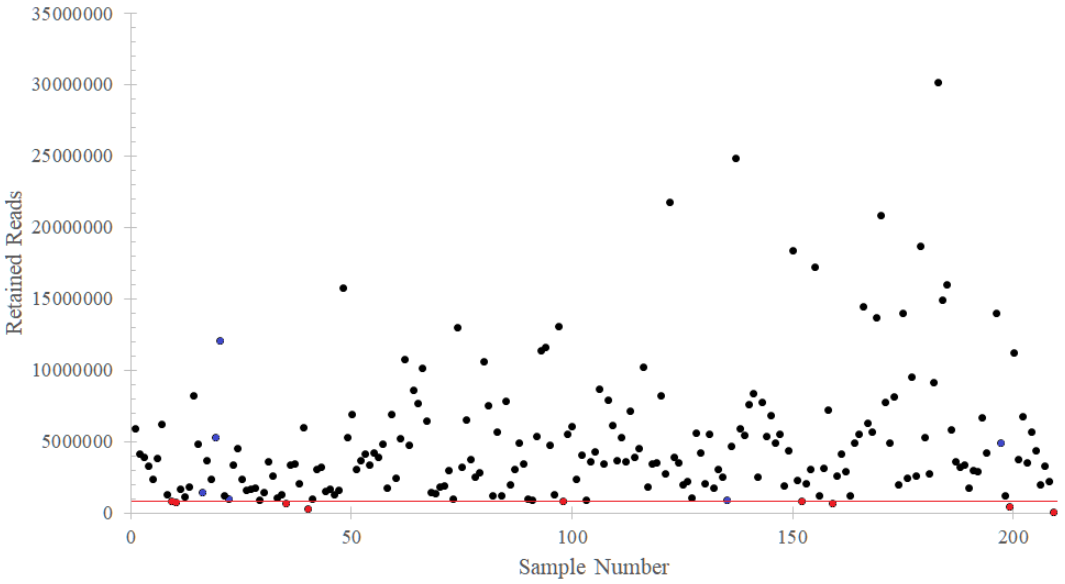

Figure 2.8: Number of retained reads per sample. Samples with less than 900,000 reads (highlighted in red, n=10) were removed from further analysis. Similarly, samples with greater than 30% missing data were also removed from further analysis (highlighted in blue, n=9). Number of samples exceeds the number utilised in this chapter as it also includes northwest Atlantic *T. truncatus* samples utilised in Chapter 4.

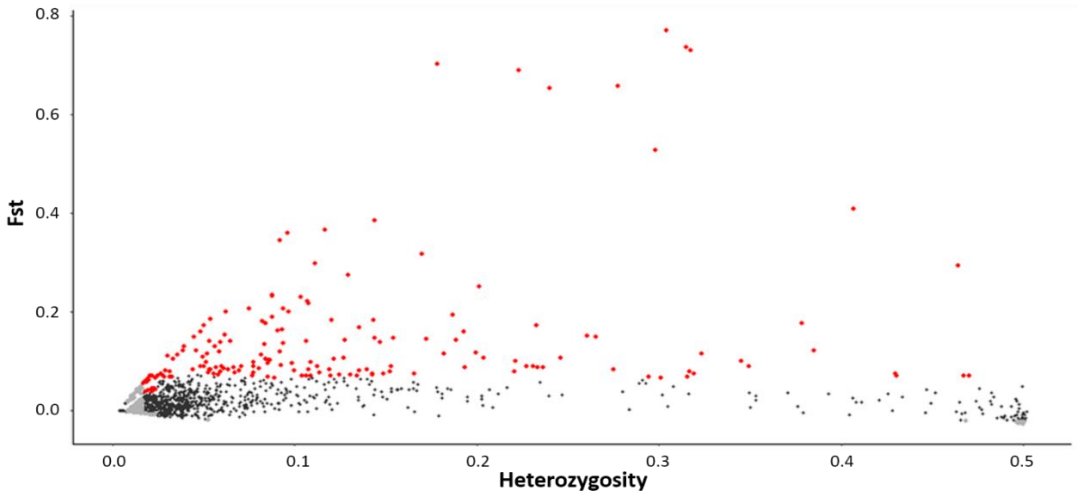

Figure 2.9: Detecting loci putatively under selection using the program *Lositan*. Loci highlighted in red (n=253) are outliers and putatively under positive selection. Loci in black (n=3476) are considered neutral and those coloured in grey (n=1375) are considered to be experiencing balancing selection

2.3.1 Genetic Diversity

East Italy had the highest proportion of segregating sites (0.0052) and the Black Sea has the lowest proportion (0.0016) (Figure 2.10A). The Black Sea displayed the highest proportion of heterozygous sites for segregating sites but the lowest when all sites were considered. West and East Italy both displayed large amounts of intrapopulation diversity for the proportion of heterozygous sites, whereas other populations were relatively consistent (Figure 2.10B&C). Investigations of Allele Frequency Spectra for each population (Figure 2.10D) indicated that the Black Sea had the highest proportion of polymorphic sites for larger Minor Allele Frequency (MAF) classes but the lowest proportion in smaller classes, whilst the reverse was true for its nearest neighbouring population, East Italy. The high proportion of intrapopulation variation in levels of heterozygous sites in East and West Italy perhaps explains the high level of rare alleles seen in these populations (Figure 2.10D). Distributions of locus-specific minor allele frequencies are displayed by population in Figure 2.10E.

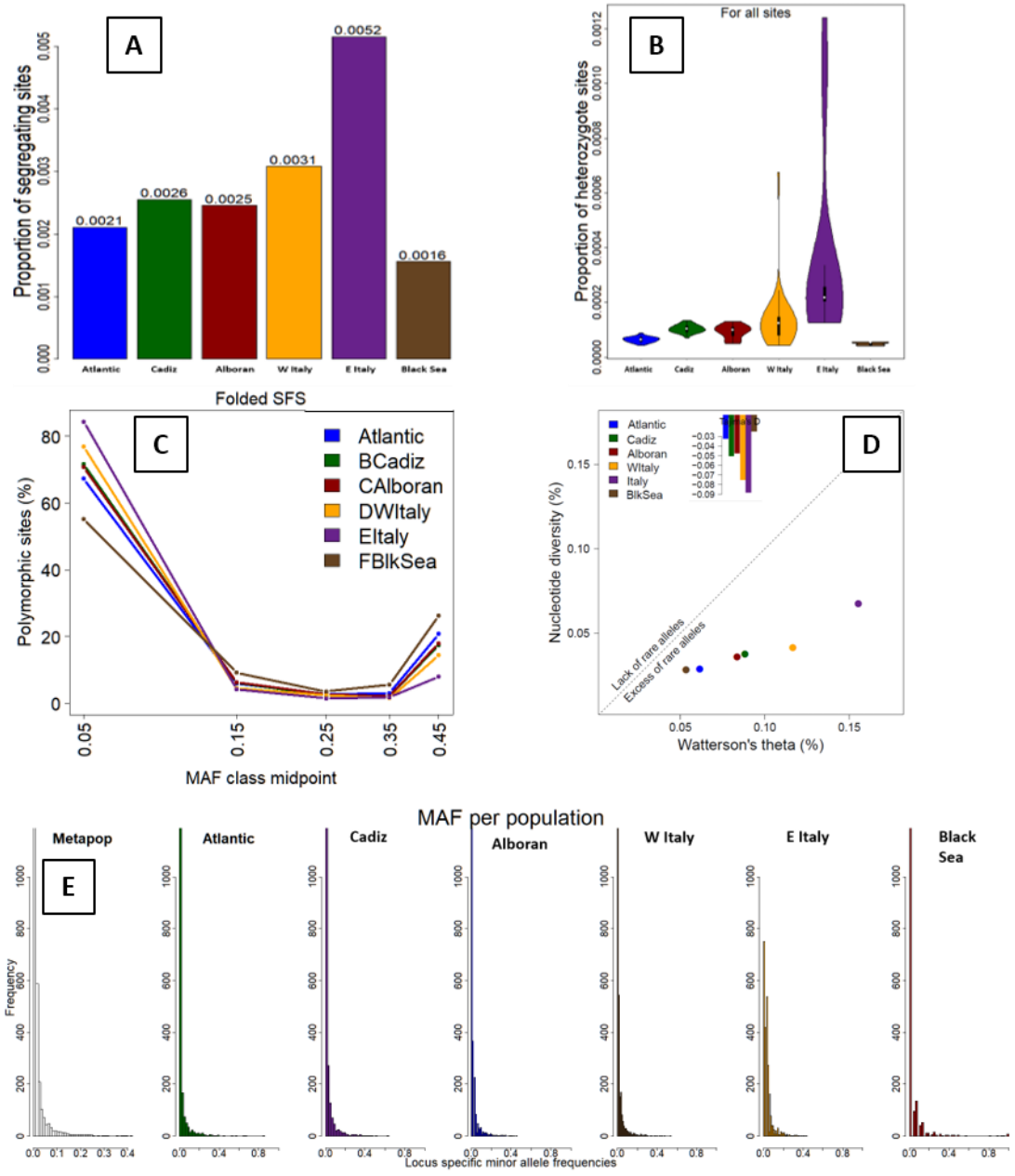

Figure 2.10: Investigations of genetic diversity between broad *a priori* populations. Analysis and output created with *SambaR*. Figure shows proportion of segregating sites (A), nucleotide diversity for all sites (B). (C) shows allele frequency spectrum per population. A comparison of Watterson's θ to nucleotide diversity shows all populations have an excess of rare alleles (D). Population Minor Allele Frequency (MAF) is shown in (E).

2.3.2 Investigating population structure

Principal Component Analysis (PCA) was run with neutral markers to try and reveal non-adaptive population structure. PCA (Figure 2.11) revealed a large area of Euclidean space shared by West Italy, East Italy and Alborán. The Atlantic formed a tight cluster, sharing some space with a few individuals from West Italy which upon inspection are revealed to be all samples from Sicily. It should be noted that individual eigenvalues account for quite a small proportion of the variation (all <4%) so it was necessary to retain a large number of principal components (n=20). Running of PCA with outlier loci, detected with *Lositan*, revealed the same spatial patterning.

Further investigation of neutral loci for evidence of population structure was conducted by constructing a distance tree based on absolute number of SNP mismatches between individuals (Figure 2.12). This revealed again a clustering of Atlantic and Sicilian individuals, forming their own similarity clade with several individuals from the Alborán population, specifically from Valencia (Valencia to Cartagena). Several individuals from West Italy and East Italy (CL59, TtTUS9, SLO1 & SLO6) formed their own outgroup, being very mismatched to all other samples. All other individuals however, formed an ambiguous clade with no clear geographic patterning.

Discriminant Analysis of Principal Components (DAPC), performed on neutral loci and retaining 40 principal components, produced a similar pattern (Figure 2.13). DAPC assignments were able to assign individuals to geographic clusters for all putative populations. Figure 2.13 reveals a cluster (right of centre) that is quite distinct from other clusters that are arranged almost linearly in Euclidean space. Inspection of the individuals that form this out-cluster reveals that it is made up of Atlantic, Sicilian and Valencian *T. truncatus*. Explorations of assignment probability

(Figure 2.14) revealed that many samples from the Sicily and Valencia regions showed a high probability of assignment to the Atlantic population. It should also be noted that Black Sea individuals cluster centrally with those individuals coming from the *a priori* defined East Italy population.

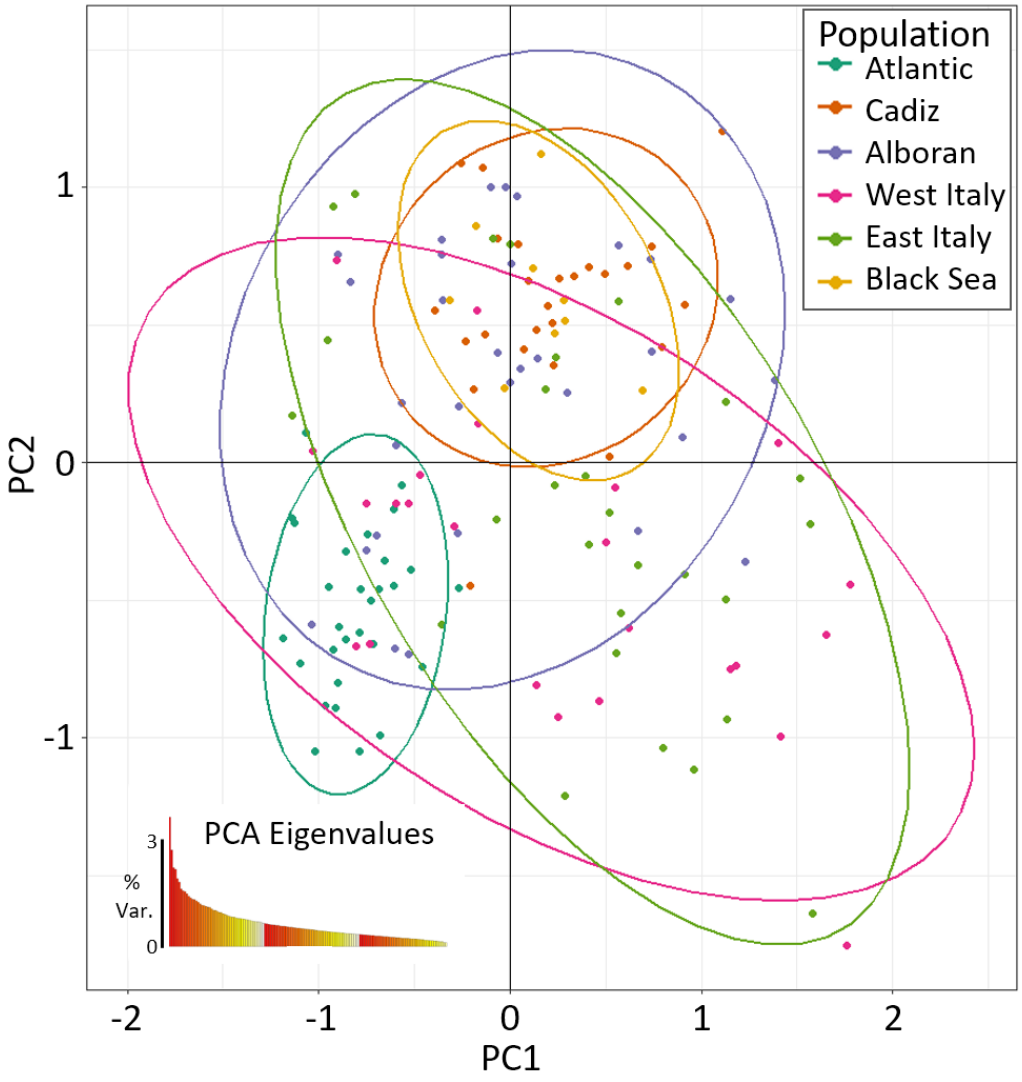

Figure 2.11: Principal component analysis of samples based on neutral loci as identified by *Lositan*. Inset shows Eigenvalues used to select the number of eigenvectors (principal components) for the Principal Component Analysis

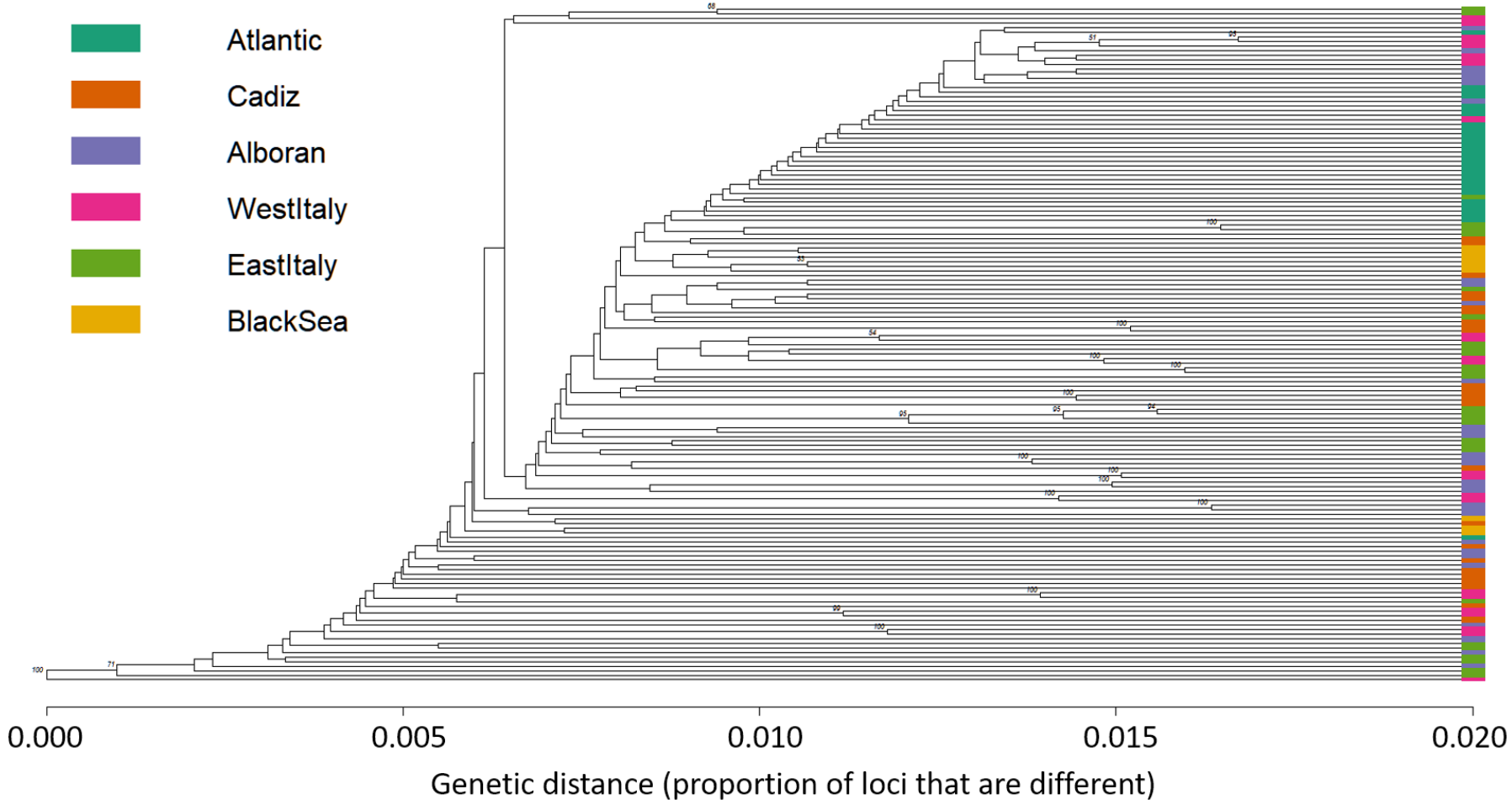

Figure 2.12: Genetic distance tree, based on Euclidean distances calculated by number of SNP mismatches between all individuals based on neutral loci as identified by *Lositan*. Note the clustering of Atlantic (Azores), Alborán (Valencia) and West Italy (Samples) in the upper half of this distance tree.

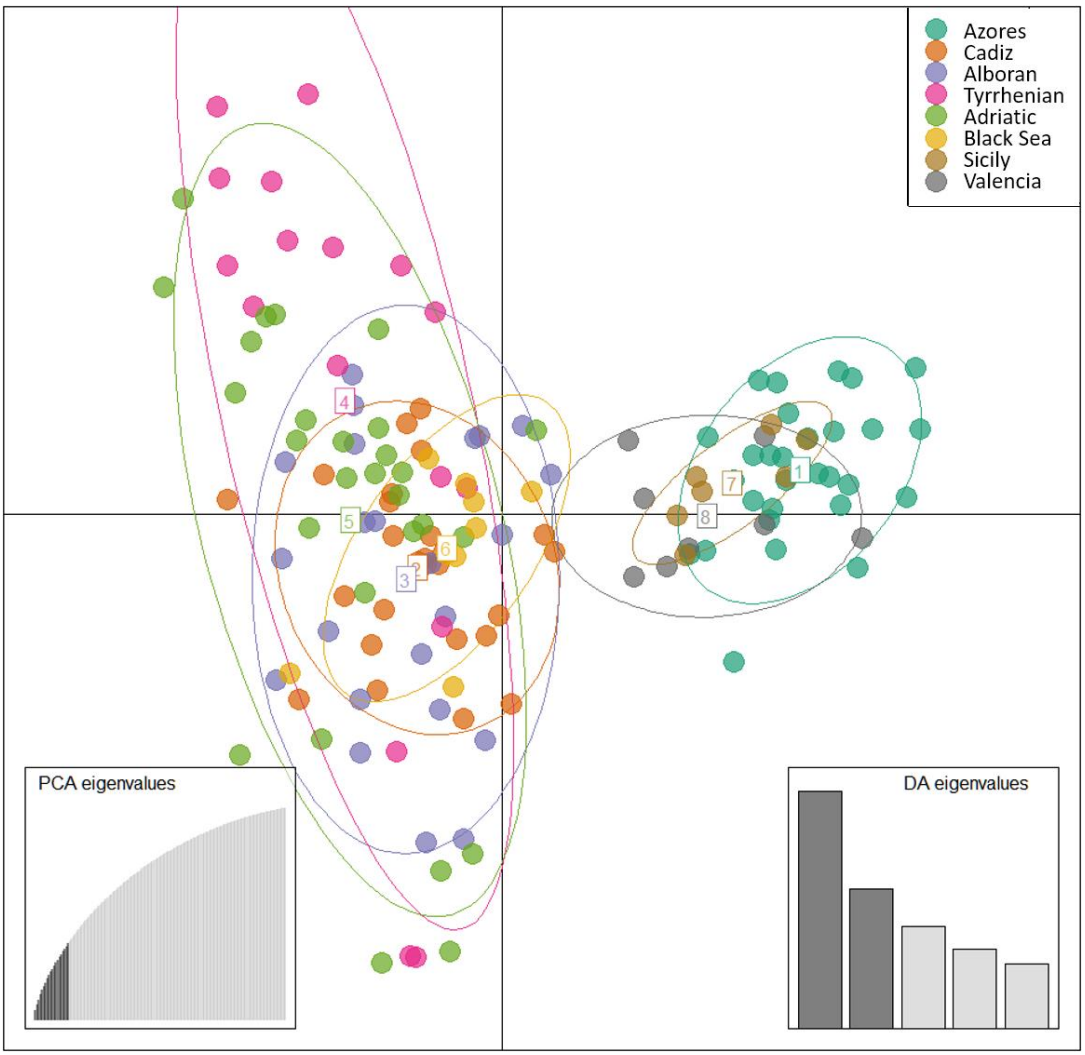

Figure 2.13: Discriminant Analysis of Principal Components based on neutral loci as identified by *Lositan*. Showing without labels (left) and with individual sample labels (right), thus clearly showing the combined clustering of Atlantic, Valencia and Sicily samples.

Investigations in to population genetic differentiation (Tables 2.1-4) revealed *T. truncatus* from the Black Sea were most distant from all other populations. Pairwise F_{ST} values based on all loci were highest between the Black Sea and the Atlantic (0.07, Table 2.1), given the geographical distance and physical marine restrictions between these two populations this makes sense.

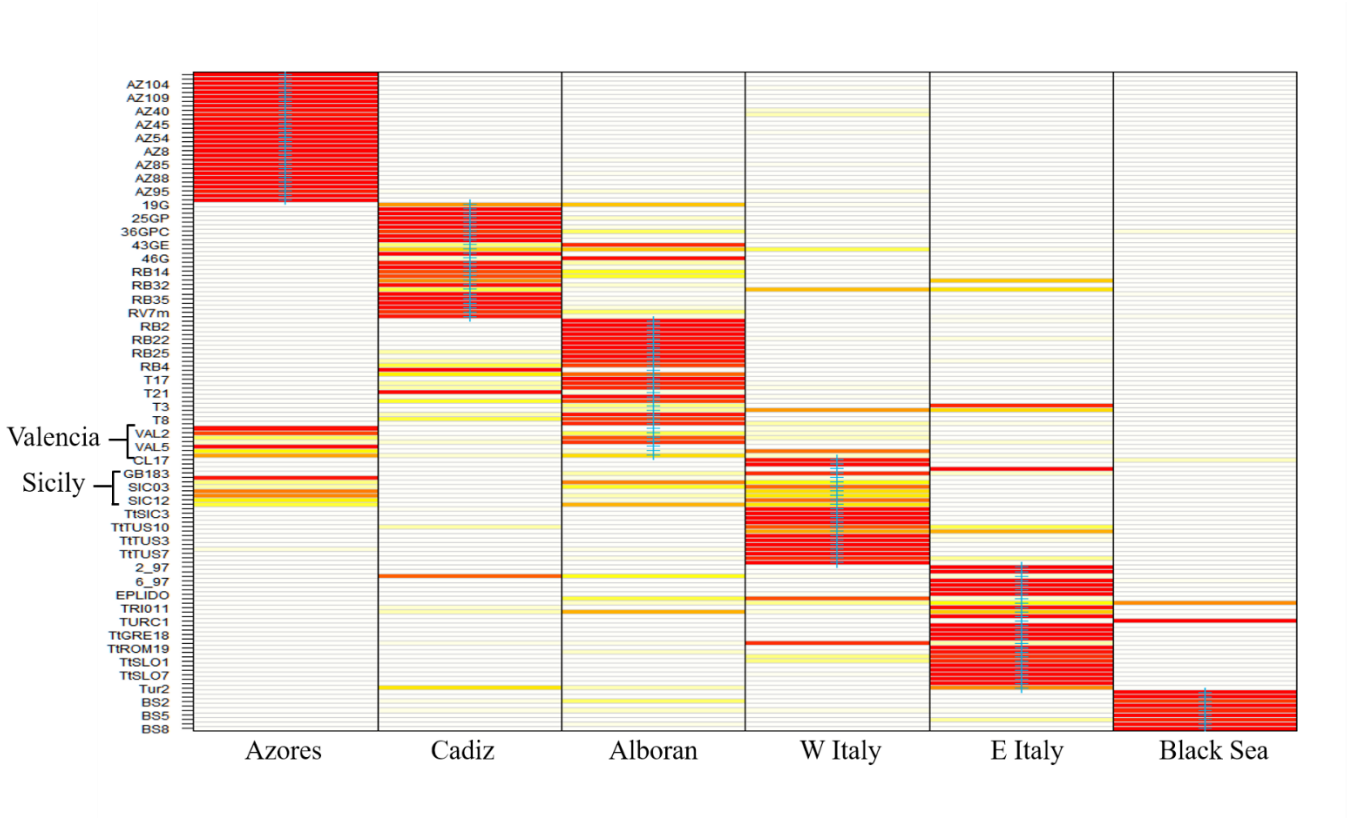

Figure 2.14: Assignment probability plot of individuals from DAPC analysis. The X-axis represents the possible assignment populations whereas the Y-axis represents the individual samples with representative sample names shown. Probabilities are represented by colour with red being high assignment probability, white being low and yellow being intermediate. The Valencia and Sicily samples are highlighted on the Y-axis and this figure clearly shows their likely assignment to the Azores (Atlantic) population.

Lowest pairwise F_{ST} values (0.009) was observed between West Italy and the *a priori* ascribed population of Alborán which includes all of Eastern Spain (Table 2.1).

Following the suggestion of genetic similarity between the Sicilian, Azorean and Valencian samples in PCA and DAPC analyses (Figures 2.11 & 2.13), estimation of the magnitude of genetic difference between these overlapping clusters was conducted by calculation of pairwise F_{ST} (Table 2.2). F_{ST} values between Sicily, Valencia and the Atlantic were all high ($\leq$0.03), indicating that these populations are still significantly differentiated, just less so than when compared to other Mediterranean populations.

As would be expected, neutral markers showed less genetic differentiation between *a priori* populations (Table 2.3, lower diagonal) than outlier loci (Table 2.3, upper diagonal). However, population differentiation patterns were consistent across both sets of loci, with Black Sea *T. truncatus* remaining the most distinct population group.

Nei's (1972) genetic distance values (Table 2.4) were also calculated for *a priori* populations through SambaR and presented the same population differentiation patterns, albeit with lower values. Interestingly, values (from all measures of genetic differentiation) between Cádiz-Alborán and Alborán-West Italy were consistently low, suggesting high levels of gene flow.

Table 2.1: Geographic population pairwise F_{ST} values calculated using *SambaR* and based on both neutral and outlier loci.

	Atlantic	Cádiz	Alborán	W Italy	E Italy	Black Sea
Atlantic	0					
Cádiz	0.025	0				
Alborán	0.033	0.013	0			
W Italy	0.03	0.02	0.009	0		
E Italy	0.065	0.058	0.028	0.022	0	
Black Sea	0.07	0.057	0.057	0.053	0.049	0

Table 2.2: Pairwise F_{ST} values based on localised genetically similar clusters (Atlantic, Valencia & Sicily) as identified by *Admixture* and *LEA* (Figure 2.15 & 2.17). N Adriatic and Black Sea are listed for comparison. F_{ST} values calculated using Arlequin 3.5 and based on outlier loci. Values labelled with * were non-significant at 0.05.

	Atlantic	Valencia	Sicily	N Adriatic	Black Sea
Atlantic	0				
Valencia	0.00004*	0			
Sicily	0.03074	-0.01028*	0		
N Adriatic	0.15523	0.06311	0.08393	0	
Black Sea	0.51332	0.40017	0.41017	0.32387	0

Table 2.3: Geographic pairwise F_{ST} values calculated using *Arlequin* v3.5 and based on neutral loci (below the diagonal) and outlier loci (above the diagonal). All values are significant at 0.05.

	Atlantic	Cádiz	Alborán	W Italy	E Italy	Black Sea
Atlantic	0	0.10726	0.0724	0.07228	0.13395	0.50516
Cádiz	0.02030	0	0.00697	0.05581	0.07674	0.36458
Alborán	0.01227	0.00251	0	0.02723	0.05639	0.37417
W Italy	0.01537	0.00834	-0.00111	0	0.04943	0.37335
E Italy	0.02399	0.01012	0.00687	0.00296	0	0.28207
Black Sea	0.03222	0.00501	0.00879	0.01029	0.01055	0

Table 2.4: Nei's genetic distance values calculated using *SambaR* and based on both neutral and outlier loci.

	Atlantic	Cádiz	Alborán	W Italy	E Italy	Black Sea
Atlantic	0					
Cádiz	0.002	0				
Alborán	0.002	0.001	0			
W Italy	0.003	0.002	0.001	0		
E Italy	0.004	0.004	0.003	0.002	0	
Black Sea	0.01	0.009	0.008	0.009	0.009	0

Investigations of population structure were conducted using the Landscape and Ecological Studies (*LEA*) package in *R* for values of *K* from 2 to 8 (Figure 2.15). K=6 was found to be the most supported hypothesis suggesting our samples can be subdivided in to six putative populations. The proportions of the coefficient of admixture of each *T. truncatus* individual's genome that originated from population *K*, for *K*=6, was then estimated in *Admixture* (Figure 2.16). Clustering in *Admixture* (Figure 2.16) revealed some clear geographically defined populations. The Black Sea clustered as a single population, suggestive of little ancestral input from other geographical regions, in support of our other analyses (F_{ST} etc.). Individuals from Greece also presented as a readily identifiable population, albeit with ancestral input from the Italian seas. Interestingly the Adriatic emerged as being split longitudinally, in to east and west Adriatic populations. Dolphins from Croatia and Slovenia clustered together in a unified Balkans population, distinct from Italian Adriatic individuals.

Individuals from the Tyrrhenian appeared to share ancestry with those of the Italian north Adriatic, in contrast of the East-West divergence seen in previous studies. It is worth noting that within the Tyrrhenian there were some individuals that are clear migrants, with strong admixture signals indicating origin from the eastern North Adriatic.

Putative populations of Cádiz, Alborán and Barcelona seemed less well-defined and are more contiguous in their mixed genetic makeup. Perhaps the most interesting result emerging from Admixture analysis however is the apparent shared ancestry between individuals from the Atlantic (Azores), Valencia and Sicily. This pattern was strongly defined for all levels of *K* investigated from 2-8 and is consistent with our previous PCA, DAPC, genetic distance tree and F_{ST} analyses.

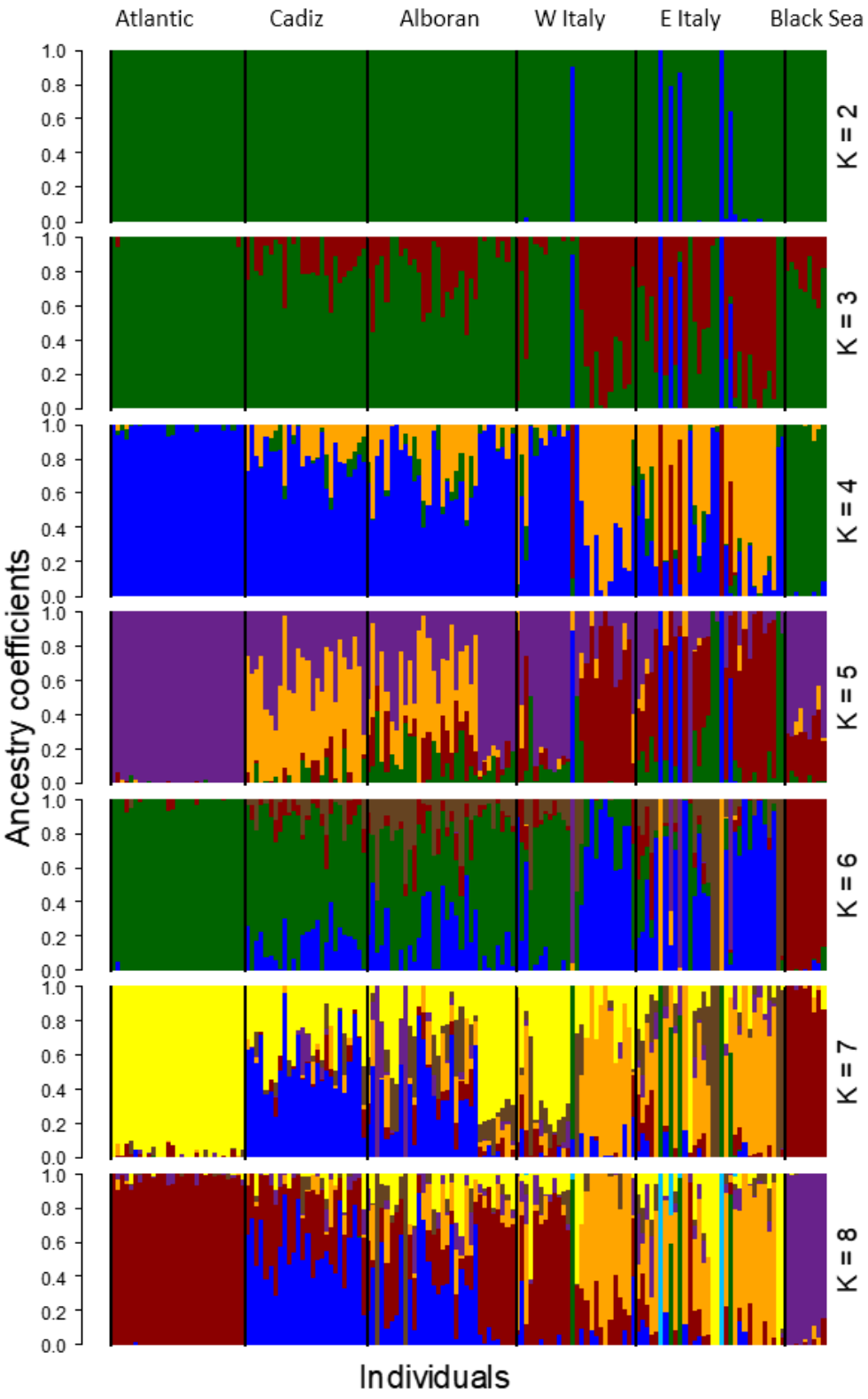

Figure 2.15: Estimated admixture coefficient for individuals across all estimates of K from 2-8. Plot derived from the *snmf* function in the *R* package *LEA*.

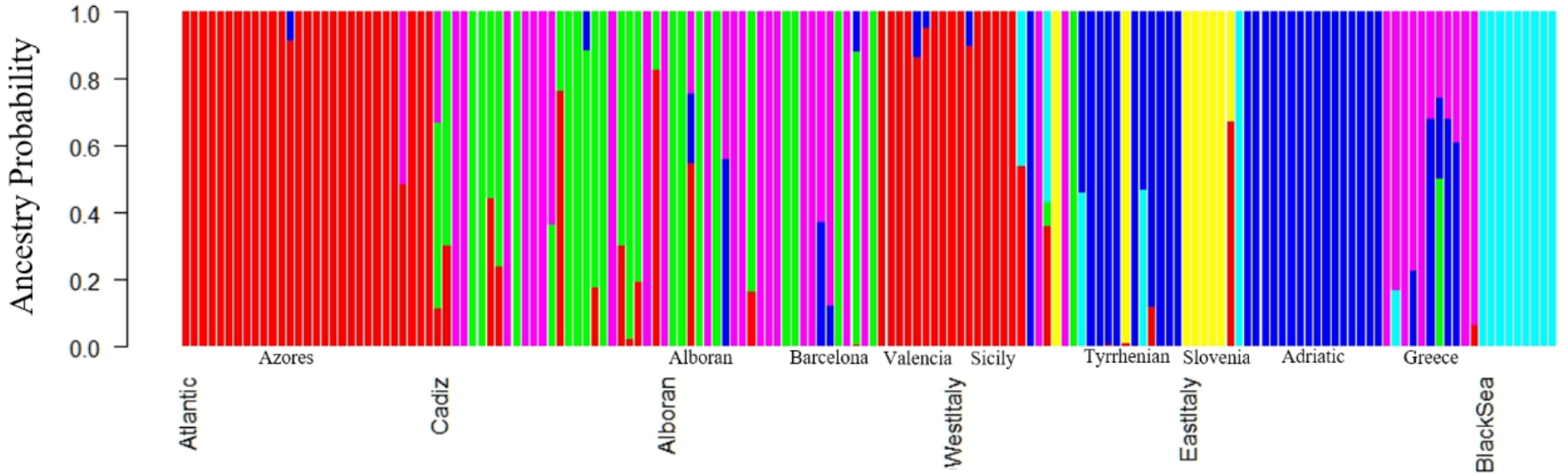

Figure 2.16: Estimated proportions of each individual's genome (admixture coefficient) that derives from hypothetical ancestral population 'K' (for K=6). Estimates developed in *Admixture* and visualised in *R*.

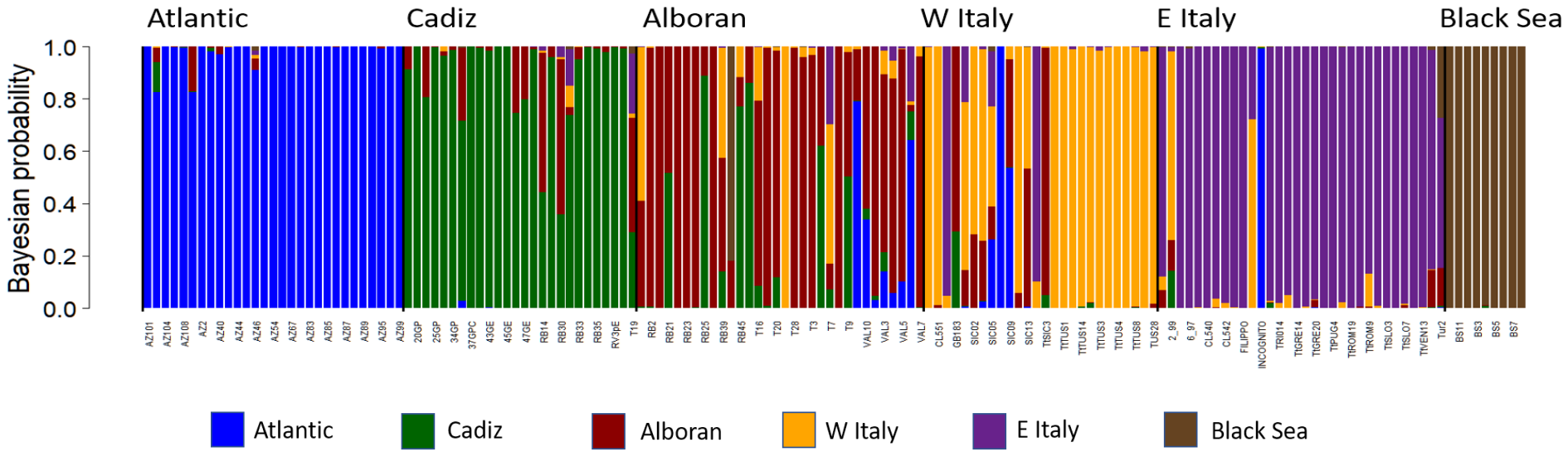

Figure 2.17: Bayesian likelihood barplot showing the probability that an individual belongs to a certain population given priors in Minor Allele Frequency. Estimations performed in *SambaR*.

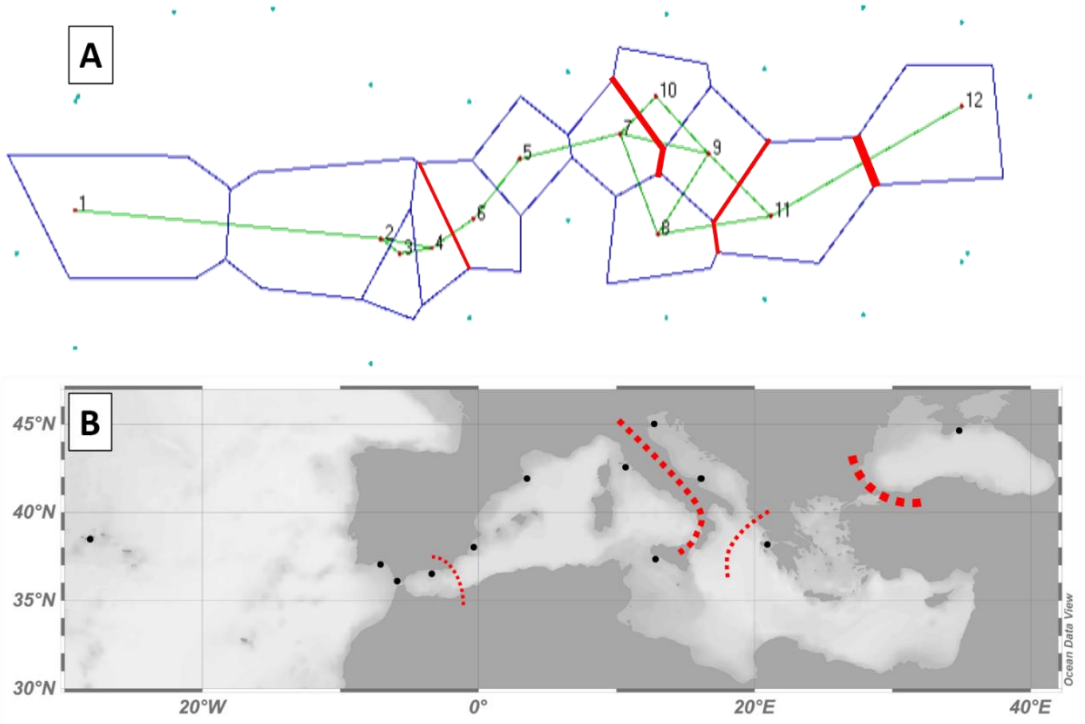

Figure 2.18: A) Projection of delauney triangulation network between localised population nodes (red dots) with the 4 strongest restrictions in gene flow, as identified through *Barrier* v2.2, highlighted by the red lines (Top). Green lines represent potential gene flow avenues to nearest neighbour populations. B) shows these results projected on to a geographical map with barriers highlighted in A) fitted to likely real-world positions.

Probability that an individual belongs to a given *a priori* population based on priors of minor allele frequency was calculated in *SambaR* (Figure 2.17). Like Admixture (Figure 2.16), *SambaR* revealed that the Black Sea was a well-defined population that was clearly differentiated from other populations, supporting its proposed status as a subspecies. It also showed that dolphins from Sicily and Valencia showed at least some probability of belonging to the Atlantic population. In contrast to *Admixture*, *SambaR* differentiated Alborán individuals from Cádiz individuals as well as Tyrrhenian from northern Adriatic. It also did not suggest any separation between Italian coast northern Adriatic dolphins from those found in Slovenian and

Croatian waters. This is to be expected, considering that this is a method with all populations prescribed *a priori* with no possibility for novel putative populations.

Testing for the strongest restriction in gene flow between populations was conducted in *Barrier* v2.2 (Figure 2.18). A Delauney triangulation network was projected between nodes set on localised geographical sample areas that had been suggested as informative of structure by any previous analysis (n=11). *Barrier* revealed that the strongest restriction in gene flow was between the Black Sea and all other populations, concurrent with our previous analyses (Figure 2.15-17) and supportive of the proposed Black Sea subspecies designation. In real terms this means that the Bosporus restricts gene flow more than any other physical environmental feature across our entire study area. *Barrier* analysis also highlighted the Apennine Peninsula as a strong barrier to gene flow with a further restriction between the Ionian and Adriatic seas. Interestingly, a fourth restriction to gene flow was also discovered that co-aligned with the Almería-Oran front.

Investigations of contemporary migration were conducted with *BayesAss3-SNPs* and visualised as circosplots through *SambaR*. Initial examination using a priori population assignments (Figure 2.19A) revealed high levels of gene flow from Alborán to all other populations except the Black Sea. Secondary levels of gene flow were high from West Italy and Atlantic to other populations. Suspecting that the pattern of contemporary gene flow indicated in Figure 2.19A was caused by the components within West Italy and Alborán (Sicily and Valencia respectively), which had shown high genetic similarity to the Atlantic population in previous analyses (Figures 2.11-17). the analysis was re-run with localised populations (Figure 2.19B). Though gene flow was apparent in all directions the greatest flow appeared to be

outward from Sicily. Secondary high levels were seen from Atlantic to other populations excluding Black Sea.

Considering the strong genetic similarity between the Atlantic, Sicily and Valencia, and taking into account other levels of genetic structure such as the longitudinal split of Adriatic dolphins, all further analyses abandon *a priori* population assignments in favour of localised *a posteriori* ones. This is especially important as I begin to investigate environmental factors as a potential driver of this structure, given that many factors vary at a much smaller scale than our *a priori* population assignments.

Historic demographies of local *a posteriori* populations are presented in Figures 2.20a-i. General trends are described below but a cautious interpretation is encouraged as some demographic events that appear correlated with environmental changes may be also created by admixture events. Admixture is known to effect demographic inference and is investigated in the discussion. The Atlantic population began a period of decline around 130,000 years ago during a period of sea level rise and inter-glacial climate. This population reached a low during the upper Pleistocene, around 50,000 years ago, then began to recover to greater than pre-decline population size where it has remained stable for at least the past 20,000 years. The Cádiz population has been in steady but staged decline for at least the last 100,000 years, a demographic pattern also displayed by the Alborán, Valencia and West Italy populations and with remarkable temporal similarity. The Sicilian sample also shows a similar pattern of staged decline but with a particularly steep and conspicuous drop in population size following the last glacial maximum, in a period of rising sea level. The Adriatic population appears to have had a sharp decline in population size followed by a rapid recovery and growth around 300,000 years ago at the end of the

Purfleet Interglacial. A long population decline followed by an only partial recovery was experienced by the Greek population with the population reaching its nadir through the Illinoian stage. Finally, the Black Sea population has been in almost continuous decline for the past 2.5 million years beginning with the first period of glaciation in the Gelasian stage.

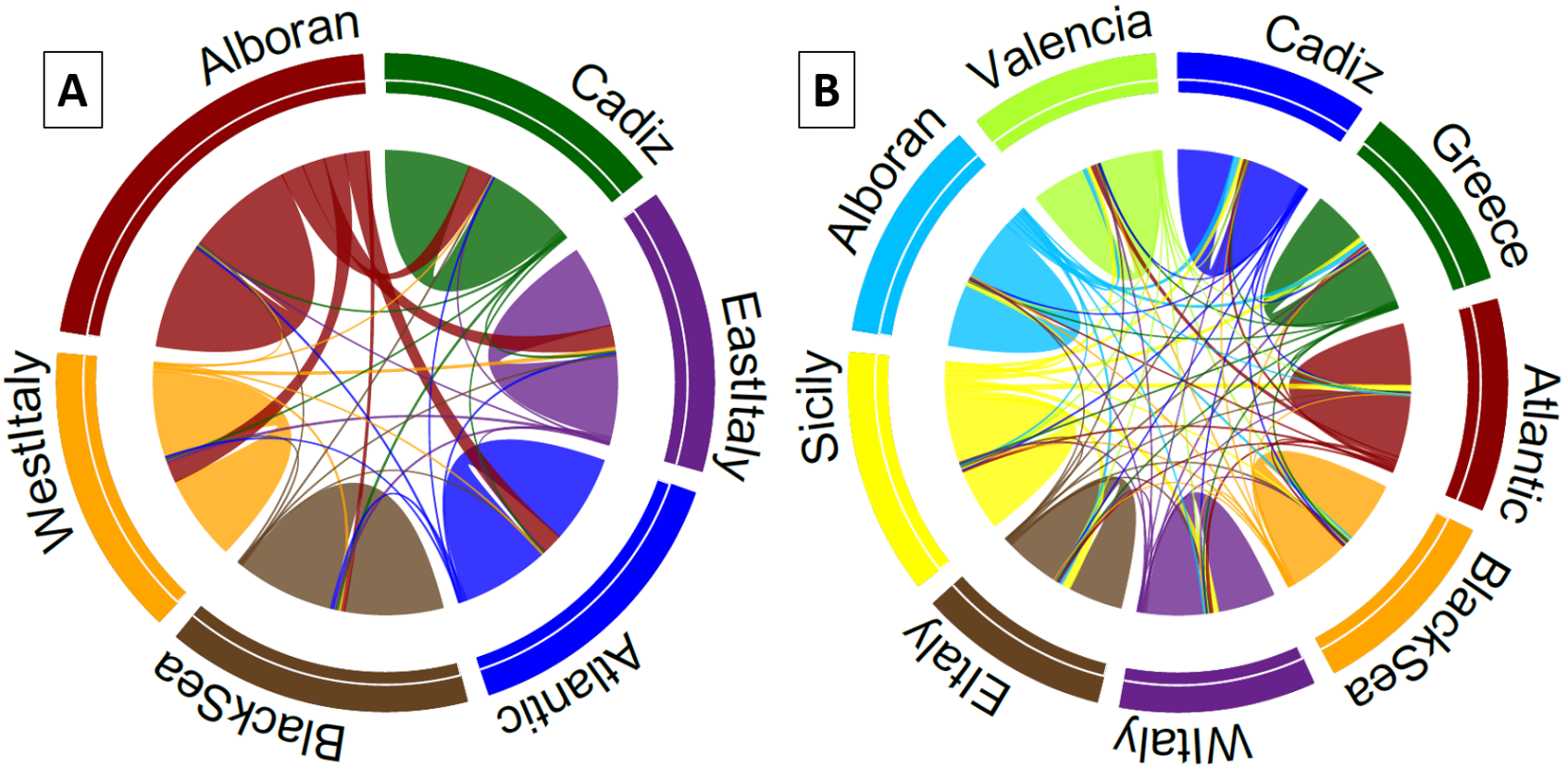

Figure 2.19: Circosplots showing migration rates between populations as calculated by *BayesAss3-SNPs*. A) shows a priori populations as defined in figure 2.8, with high levels of gene flow from Alborán to other areas and secondarily from West Italy and Atlantic. B) shows more locally defined populations with high levels of gene flow from Sicily and secondarily from the Atlantic.

Table 2.5: Inferred posterior mean migration rates as calculated in *BayesAss3-SNPs* shown in Figure 2.19A. Migration rates (bold) can be interpreted as fraction of individuals in row population that are migrants derived from column population. Values underneath represents 95% CI set when ± to mean value.

	Cádiz	East Italy	Atlantic	Black Sea	West Italy	Alborán
Cádiz	**0.759**	**0.010**	**0.010**	**0.011**	**0.010**	**0.200**
±	0.093	0.020	0.019	0.020	0.020	0.095
East Italy	**0.017**	**0.724**	**0.017**	**0.009**	**0.035**	**0.198**
±	0.024	0.056	0.024	0.017	0.035	0.060
Atlantic	**0.010**	**0.010**	**0.782**	**0.009**	**0.009**	**0.180**
±	0.018	0.019	0.176	0.018	0.018	0.177
Black Sea	**0.022**	**0.022**	**0.022**	**0.875**	**0.023**	**0.037**
±	0.040	0.040	0.040	0.082	0.042	0.055
West Italy	**0.011**	**0.021**	**0.010**	**0.011**	**0.759**	**0.188**
±	0.020	0.028	0.020	0.020	0.070	0.074
Alborán	**0.015**	**0.009**	**0.015**	**0.009**	**0.009**	**0.943**
±	0.023	0.016	0.024	0.017	0.017	0.042

Table 2.6: Inferred posterior mean migration rates as calculated in *BayesAss3-SNPs* shown in Figure 2.19B. Migration rates (bold) can be interpreted as fraction of individuals in row population that are migrants derived from column population. Values underneath represents 95% CI set when ± to mean value.

	Cádiz	Greece	Atlantic	Black Sea	West Italy	East Italy	Sicily	Alborán	Valencia
Cádiz	**0.770**	**0.010**	**0.034**	**0.010**	**0.010**	**0.010**	**0.052**	**0.095**	**0.010**
±	0.083	0.019	0.043	0.018	0.018	0.019	0.049	0.078	0.018
Greece	**0.019**	**0.753**	**0.026**	**0.019**	**0.019**	**0.018**	**0.068**	**0.060**	**0.018**
±	0.035	0.091	0.043	0.035	0.034	0.033	0.074	0.070	0.034
Atlantic	**0.009**	**0.009**	**0.851**	**0.009**	**0.009**	**0.009**	**0.088**	**0.009**	**0.009**
±	0.016	0.017	0.102	0.017	0.016	0.017	0.099	0.016	0.016
Black Sea	**0.018**	**0.019**	**0.018**	**0.839**	**0.018**	**0.019**	**0.018**	**0.029**	**0.023**
±	0.033	0.034	0.032	0.075	0.034	0.036	0.034	0.046	0.040
West Italy	**0.014**	**0.014**	**0.036**	**0.014**	**0.793**	**0.024**	**0.078**	**0.014**	**0.014**
±	0.026	0.026	0.060	0.025	0.104	0.036	0.065	0.025	0.027
East Italy	**0.011**	**0.013**	**0.016**	**0.010**	**0.027**	**0.750**	**0.123**	**0.039**	**0.011**
±	0.020	0.024	0.027	0.019	0.033	0.057	0.061	0.038	0.021
Sicily	**0.018**	**0.016**	**0.022**	**0.017**	**0.016**	**0.017**	**0.861**	**0.017**	**0.017**
±	0.033	0.031	0.039	0.032	0.029	0.031	0.072	0.031	0.031
Alborán	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	**0.012**	**0.059**	**0.856**	**0.012**
±	0.023	0.024	0.023	0.022	0.023	0.023	0.056	0.068	0.023
Valencia	**0.013**	**0.014**	**0.059**	**0.014**	**0.014**	**0.014**	**0.060**	**0.062**	**0.749**
±	0.025	0.025	0.058	0.027	0.027	0.026	0.065	0.085	0.138

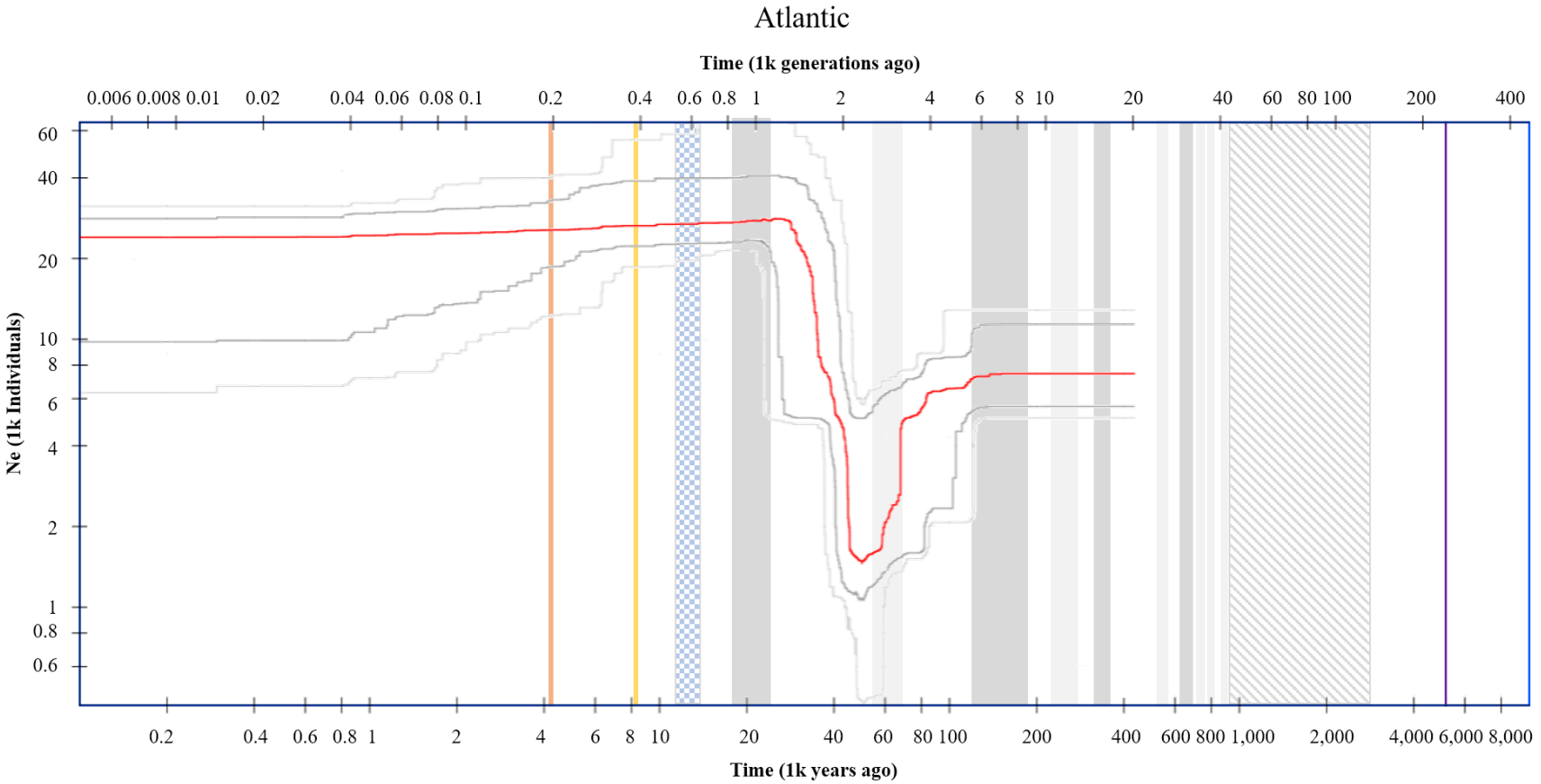

Figure 2.20a: Demography of the Atlantic population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

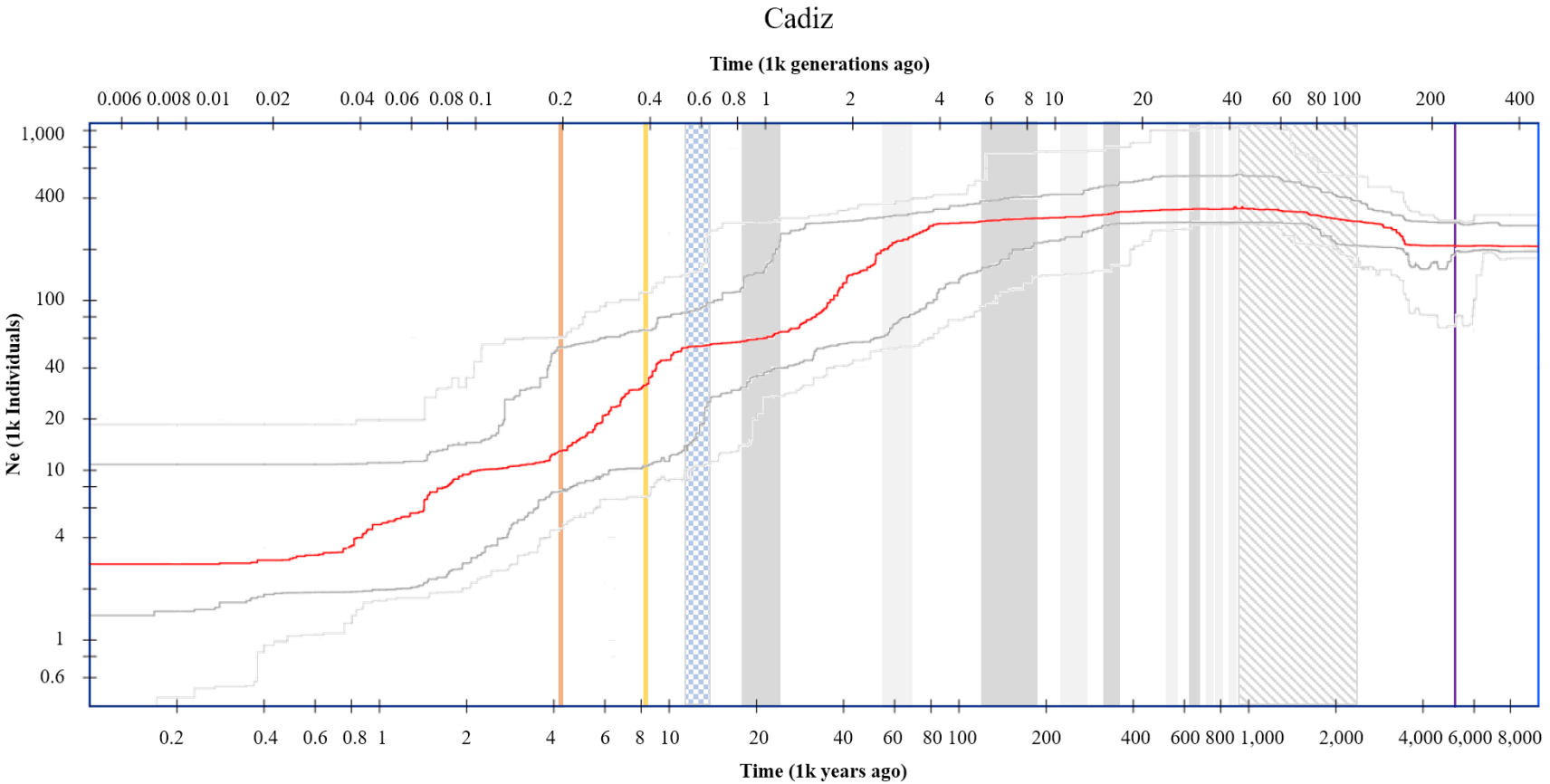

Figure 2.20b: Demography of the Cádiz population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

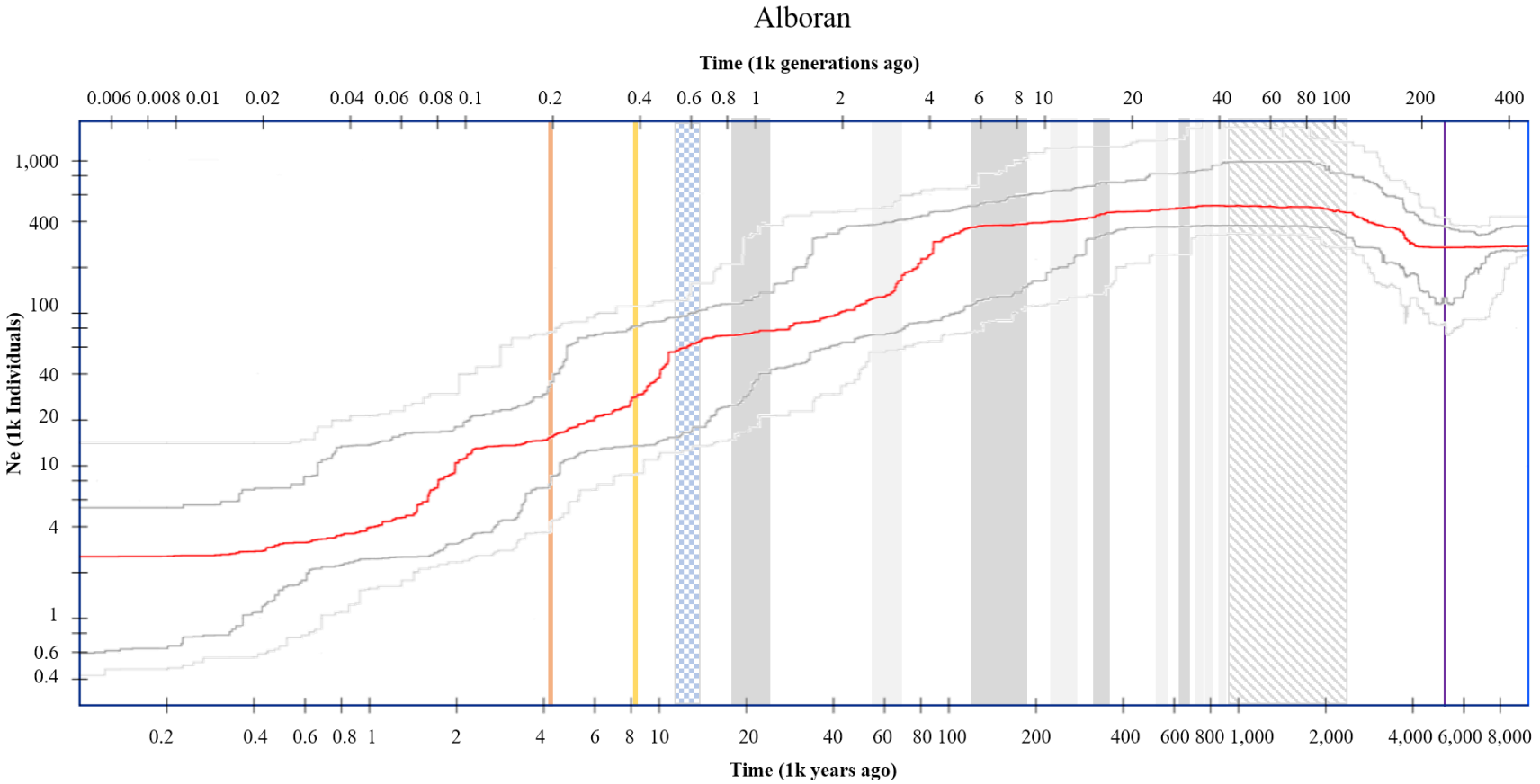

Figure 2.20c: Demography of the Alborán population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

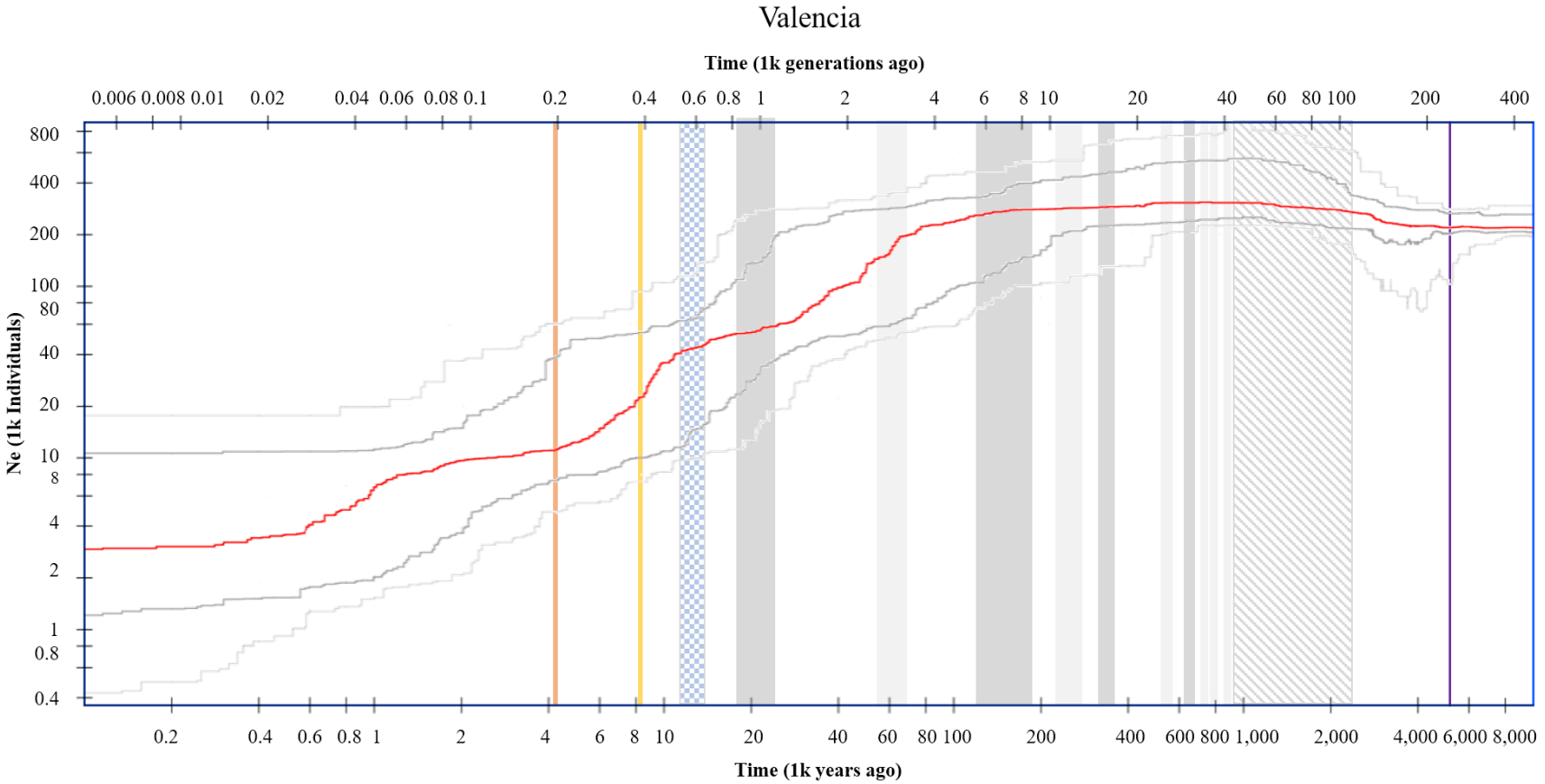

Figure 2.20d: Demography of the Valencia population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

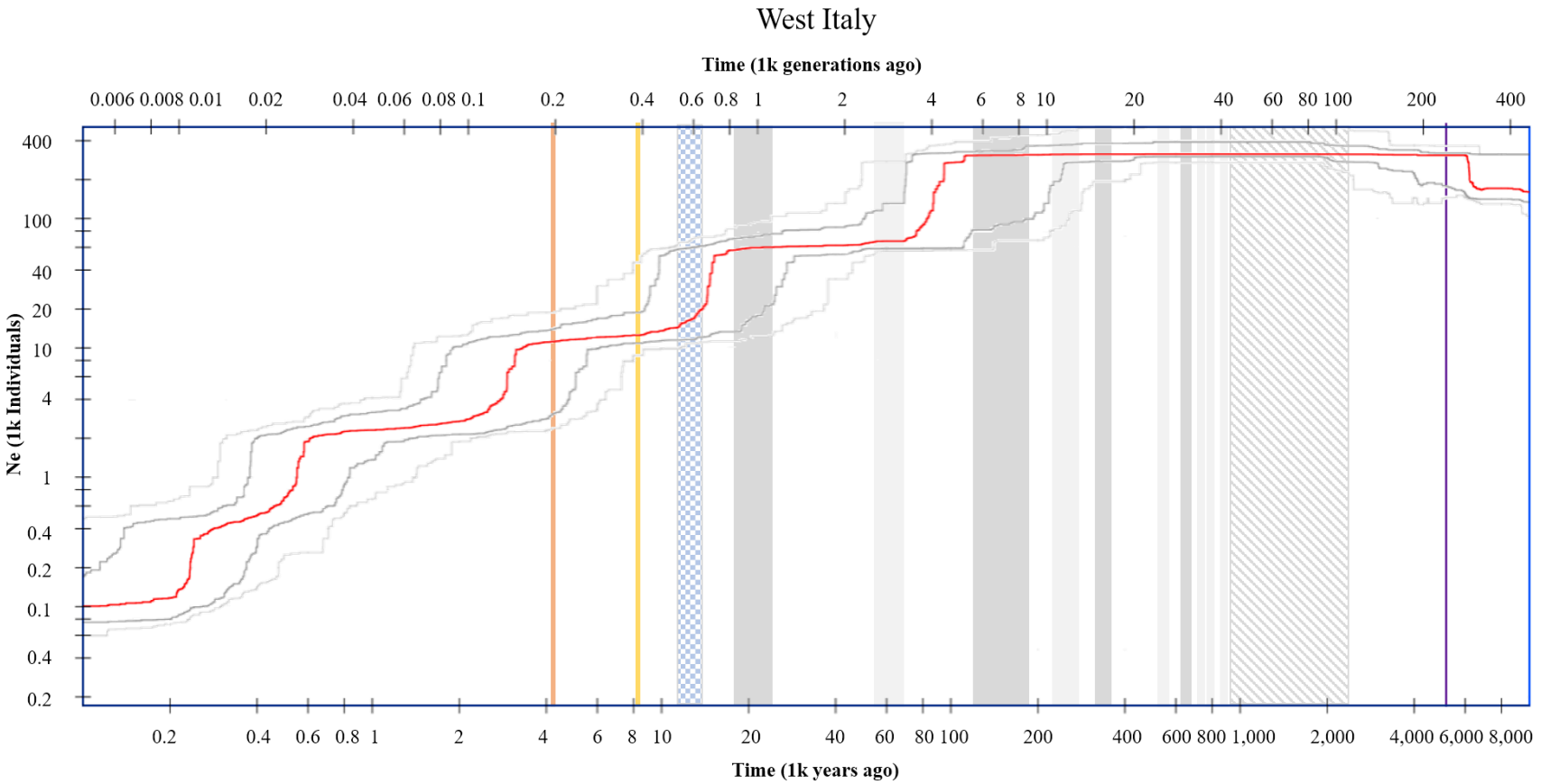

Figure 2.20e: Demography of the West Italy population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

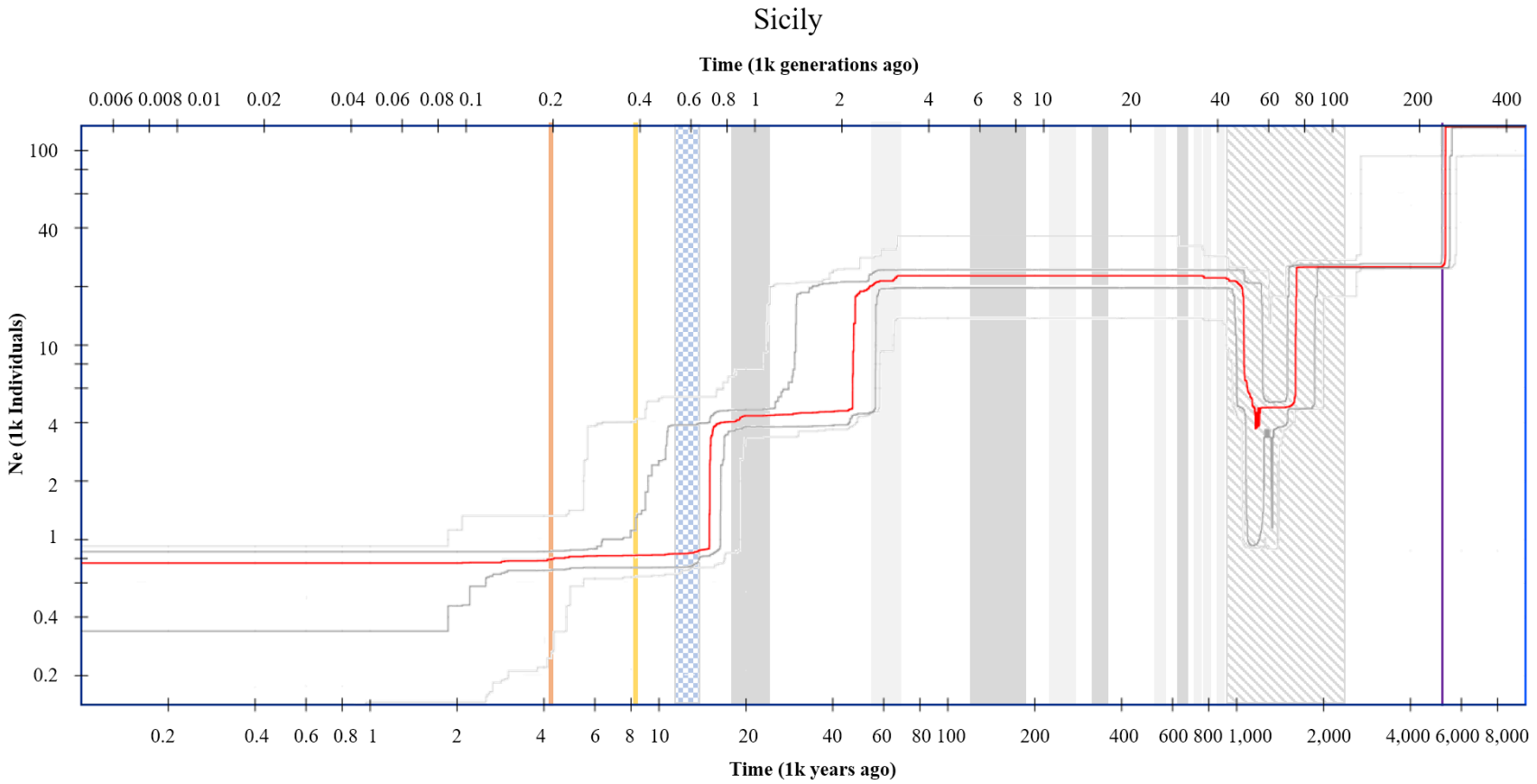

Figure 2.20f: Demography of the Sicilian population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

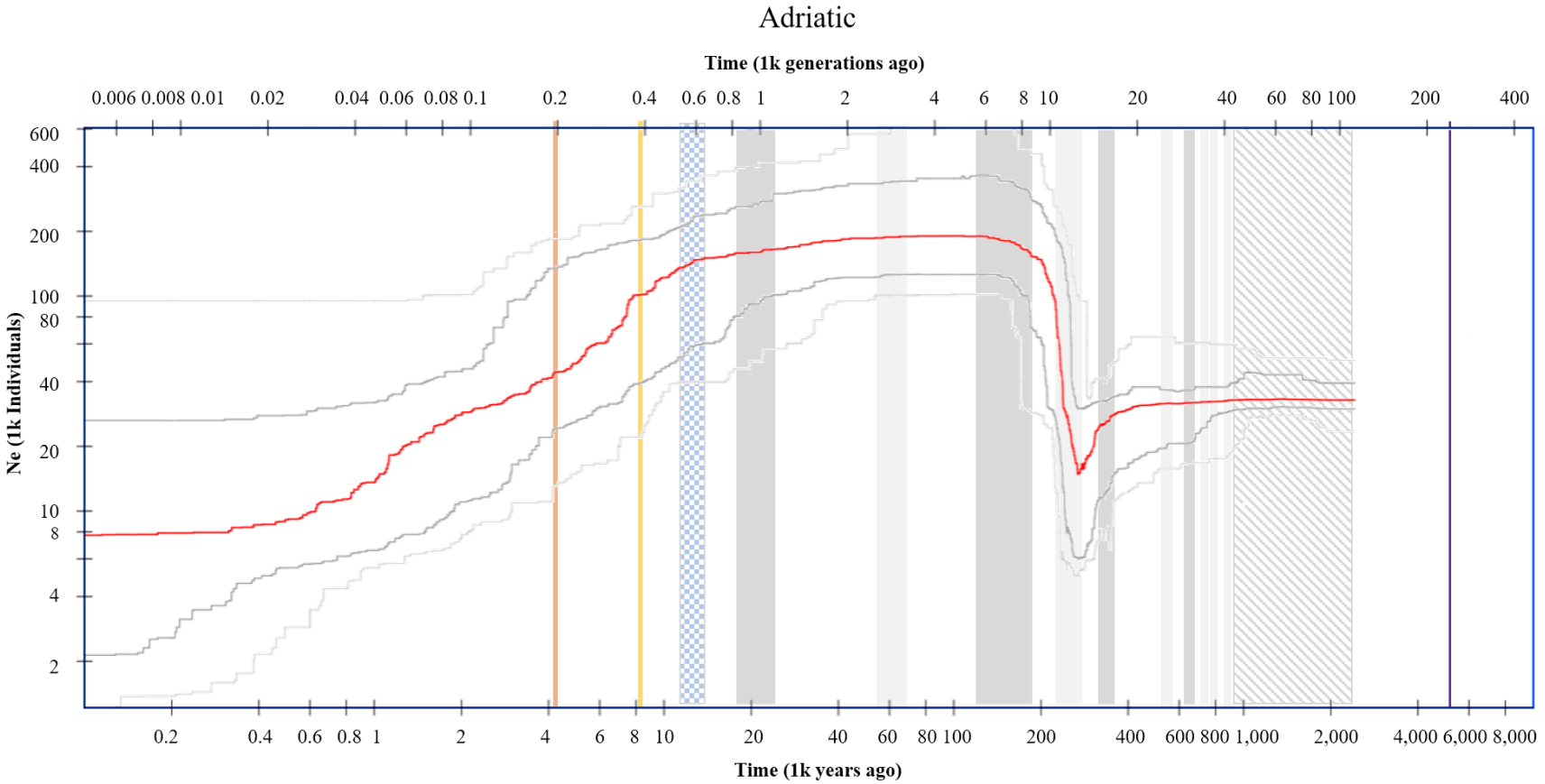

Figure 2.20g: Demography of the Adriatic population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

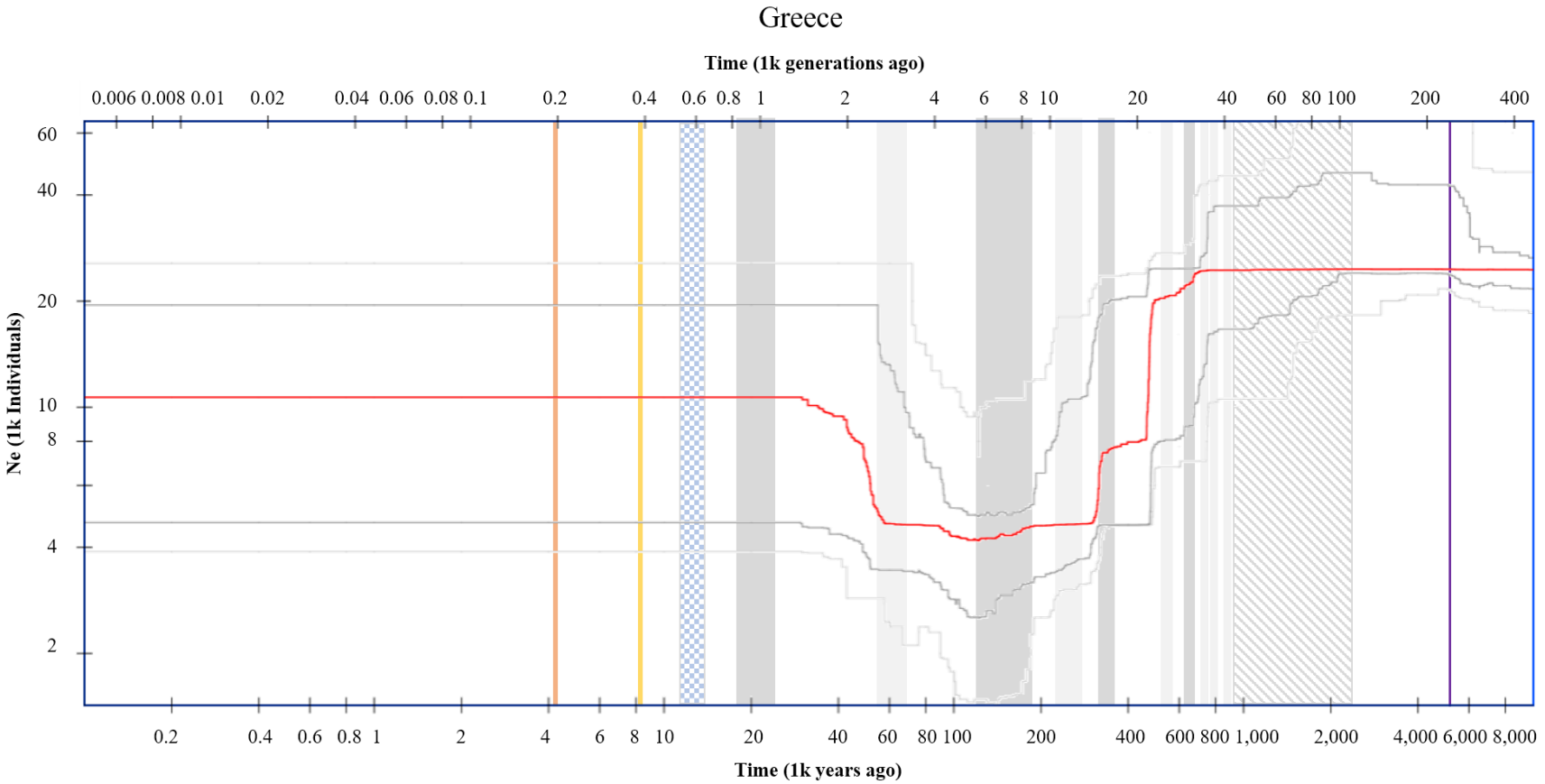

Figure 2.20h: Demography of the Greek population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

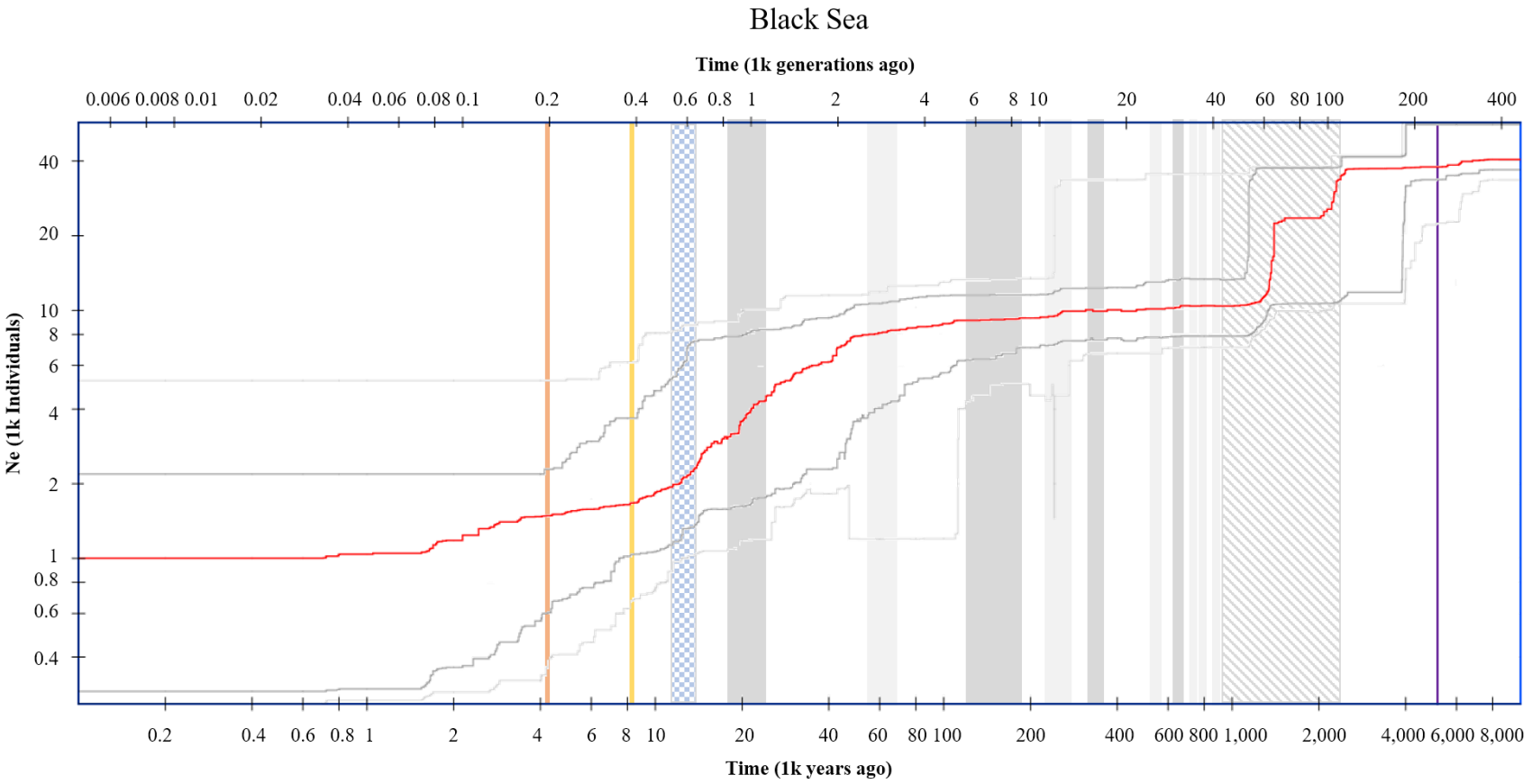

Figure 2.20i: Demography of the Black Sea population as derived from *ANGSD* calculated SFS and annotated with various climate events of the past 8 million years. Vertical grey bars indicate glacial periods (shading indicates intensity with darker shading illustrating more intense periods of glaciation). The glacial cycles of the Gelasian and early Calabrian are too fine to show on this scale and so this whole period is illustrated by the vertical block with diagonal lines. The Younger-Dryas event is indicated by the blue and white chequered vertical bar. The 4.2 kiloyear event, 8.2 kiloyear event and Zanclean flood are indicated by the orange, yellow and purple vertical lines respectively.

2.3.3 Exploration of environmental and dietary factors influencing population structure

Initial investigations into the potential drivers of the observed population structure began with a consideration of dietary and trophic factors through stable isotopes. Stable isotope values were available for 75 individuals and came from multiple tissue types (see Appendix V for details). To allow for this samples from the same location (Azores – Figure 2.21A) with two tissue types, skin and muscle, were tested for differentiation (Figure 2.21B) to investigate the possibility of data transformation. Values for $\delta^{15}N$ did not meet parametric test assumptions (data not normal, Anderson-Darling test, p=0.041) so were tested with the non-parametric Kruskal-Wallis test. Means were found to be not significantly different (Kruskal-Wallis, p=0.631). Values for $\delta^{13}C$ met parametric assumptions (Anderson-Darling test, p=0.715 and 0.154 for skin and muscle respectively, Levene's test, p=0.824) and were also found to not be significantly different (T test, p=0.297).

Although this investigation indicates that cross-tissue comparison could be possible it is based on a necessarily small sample size, due to sample availability, and the literature indicates that different tissues can give large variation in values. Paucity of samples in Figure 2.21A suggests a reliable data transformation (such as performed in Chapter 3 – see section 3.3.2) cannot be conducted as there is not enough data to give a clear relationship. This is further complicated by samples having unknown tissue types (n=15), including for whole geographic regions (Cádiz) as well as the complexity created by numerous samples (n=7) being from a variety of organ tissues. For this reason, isoscape maps (Figure 2.24-25) were produced conservatively using only SI values derived from skin samples (n=29).

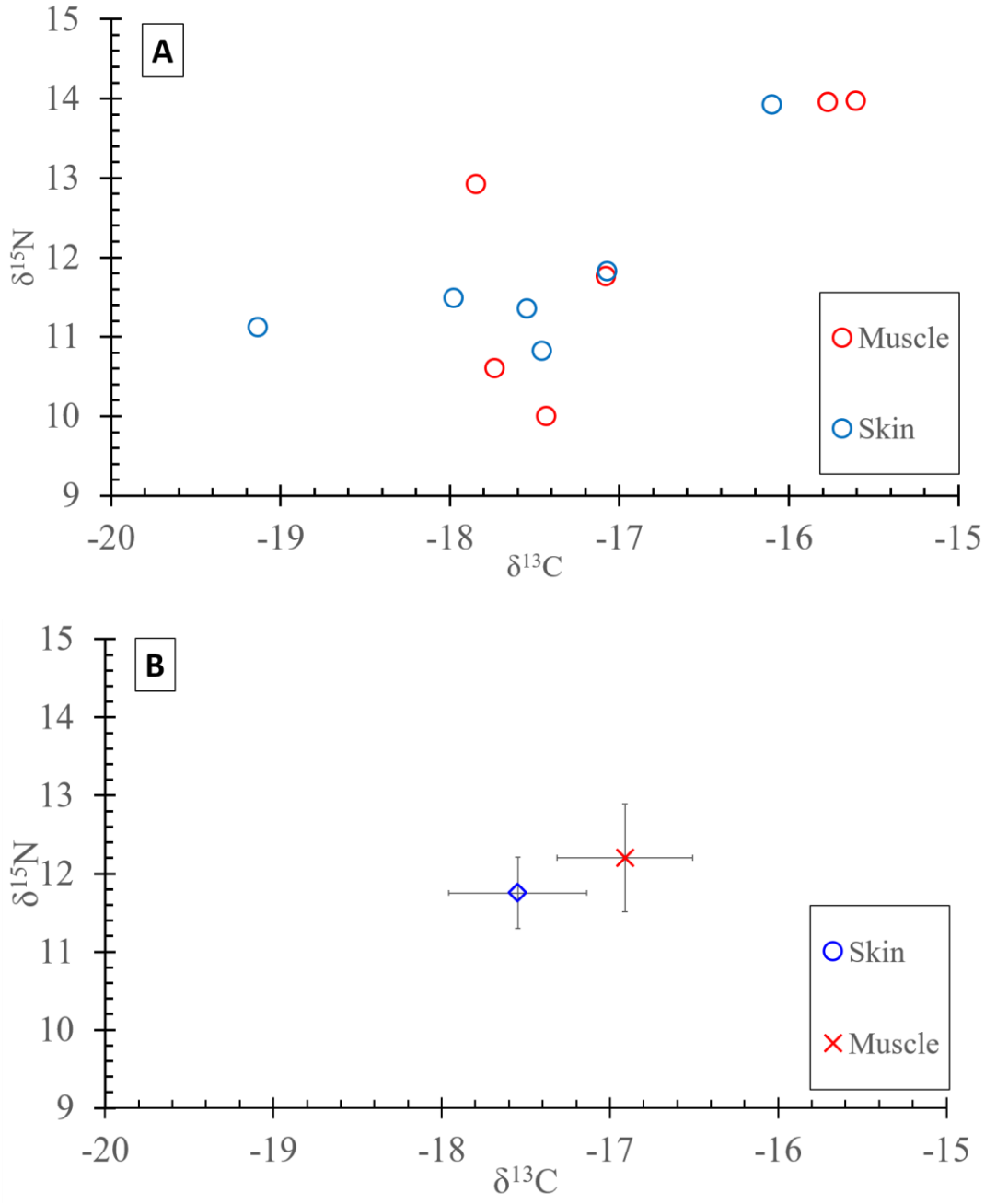

Figure 2.21: Stable isotope samples taken from the same location (Azores) were compared between two tissue types (skin, n=6 and muscle, n=6). $\delta^{13}C$ and $\delta^{15}N$ values for individual samples are shown in A and tissue means in B. Error bars are equal to one standard error.

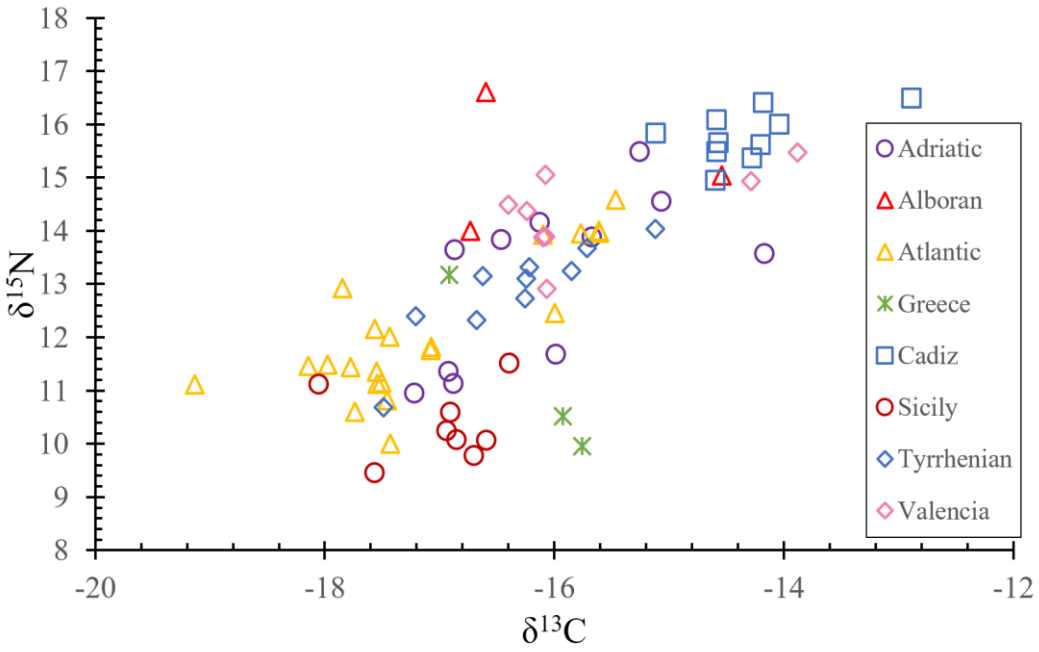

Figure 2.22: $\delta^{13}C$ values vs $\delta^{15}N$ values for all samples, including a mix of tissue types. Values closer to the top right would indicate feeding at a higher trophic level than those found at the bottom left.

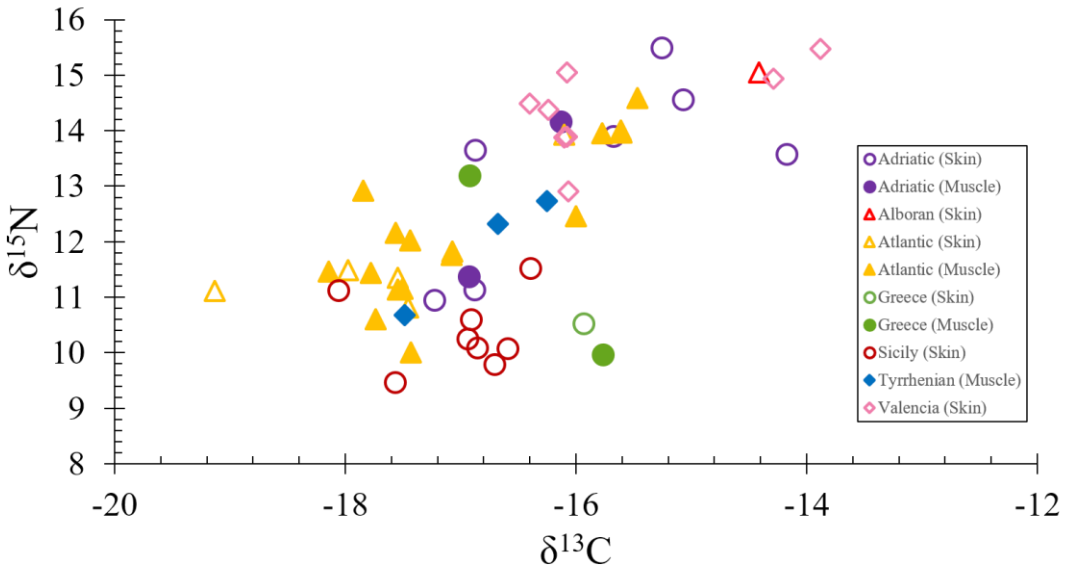

Figure 2.23: $\delta^{13}C$ values vs $\delta^{15}N$ values for skin and muscle samples only. Skin samples are represented by open symbols whereas muscle samples are represented by closed symbols.

When $\delta^{15}N$ values are plotted against values for $\delta^{13}C$ (Figure 2.22) there is suggestion that *T. truncatus* individuals from the Bay of Cádiz were feeding at a higher trophic level than other groups. By contrast, most Atlantic and Sicilian *T. truncatus* were feeding at a lower trophic level. Most other populations had significant intra-population noise with individuals positioned over a broad spectrum of trophic levels. *T. truncatus* from Greece appear to be feeding at a similar trophic level to Atlantic individuals but likely have less negative $\delta^{13}C$ values due to the higher salinity found in the eastern Mediterranean. Similarly, the less negative $\delta^{13}C$ values exhibited by individuals from the Bay of Cádiz is indicative of the coastal environment in which they inhabit, when compared to individuals from the pelagic environment of the Azores.

However, these interpretations should be treated with caution as these are derived from multiple tissue types and some of unknown origin. However, examination of data that derive only from either skin or muscle samples (Figure 2.23) appears to show no obvious dichotomy between tissue types so, except for the unknown origin of Cádiz samples, inferences are likely to be realistic.

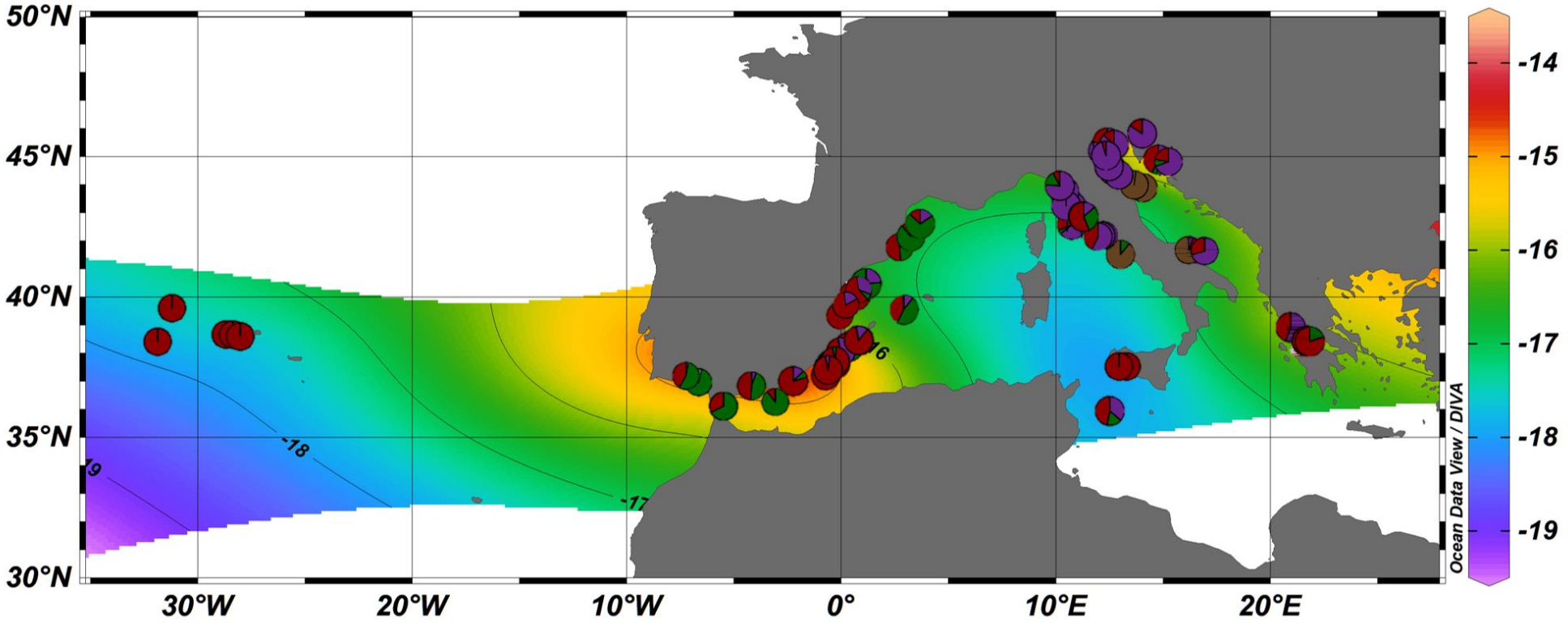

Figure 2.24: Isoscape for $\delta^{13}C$ generated from skin only samples of *Tursiops truncatus*. Pie charts represent genetic structure as informed by Admixture. Isoscape plotted using *Ocean Data View*.

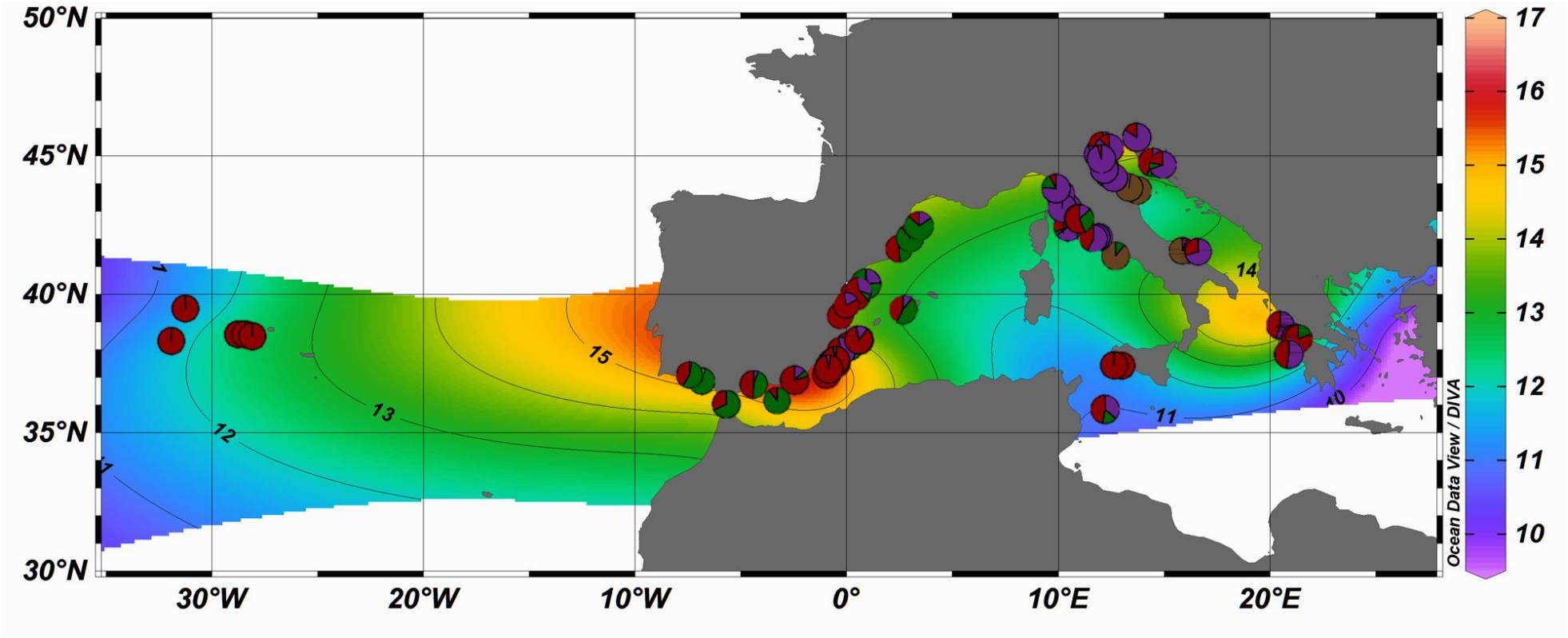

Figure 2.25: Isoscape for $\delta^{15}N$ generated from skin only samples of *Tursiops truncatus*. Pie charts represent genetic structure as informed by Admixture. Isoscape plotted using *Ocean Data View*.

The $\delta^{13}C$ isoscape (Figure 2.24) depicts the classic understanding of higher values in coastal waters and lower values in pelagic habitats. All coastal waters had a $\delta^{13}C$ value of -16‰ whereas some pelagic environments were around -18‰. Particularly high values (-14.7‰), indicating enrichment of ^{13}C, were observed around the coast of Spain, in particular the north coast of the Alborán Sea. Coastal waters of the Balkans also featured high values (≈ -15.5‰).

The isoscape for $\delta^{15}N$ (Figure 2.25) revealed a remarkably similar visual pattern to that of $\delta^{13}C$ (Figure 2.24). Higher values, indicative of greater enrichment for ^{15}N and feeding at higher trophic levels, were seen in coastal waters throughout the study area. Again, the highest values were seen around the Iberian Peninsula, in particular the north coast of the Alborán Sea. There was an interesting anomaly observed in the southern Adriatic, with particularly high values (14.5‰) seen between the Italian region of Puglia and the Greek coast.

Actual $\delta^{13}C$ and $\delta^{15}N$ values and isoscape derived values are used for subsequent analyses. Further environmental data come from a variety of sources. Sea Surface Temperature (SST) comes from CNR-Med satellite data (Nardelli *et al*., 2013). Salinity data are derived from the Copernicus Marine Environment Monitoring Service (von Schuckmann *et al*., 2016). Chlorophyll A data are CNR processed SeaWiFS satellite data (Gregg and Casey, 2004).

Initial investigations of environmental drivers of the observed population structure was conducted via Mantel tests based on matrices of genetic distance and individual environmental factors (Table 2.7). Only the (positive) correlation between salinity and genetic distance was significant (P=0.004). All other tests (distance, SST, Chlorophyll A, $\delta^{13}C$ and $\delta^{15}N$) returned non-significant correlations at the 0.05 level.

Table 2.7: Results of Mantel tests of correlation between matrices of Nei's genetic D and environmental variable matrices. Salinity is the only matrix significantly correlated with genetic distance.

Env. variable	Mantel Statistic	P-value	Significant?
Geographic distance	0.4672	0.101	N
SST	0.2931	0.091	N
Salinity	**0.9762**	**0.004**	Y
Chlorophyll A	-0.1526	0.804	N
$\delta^{13}C$	-0.3718	0.956	N
$\delta^{15}N$	0.0880	0.322	N

Redundancy Analysis (RDA) was first carried out on neutral loci only (Figure 2.25A). As would be expected from loci not thought to be under selection, environmental factors only explained a very low proportion of the total genetic variance (1.2%) but this explanation was significant ($p<0.001$). Of the components examined, RDA1 explained the most variance (0.28%), of which the factor SST makes the largest contribution. This was closely followed by RDA2 (0.27%) for which salinity was the largest contributor. Only variances explained by RDA1, RDA2 and RDA3 were significant ($p<0.05$). No factors showed evidence of collinearity through investigation of variance inflation in *R* package *Vegan*.

RDA of outlier loci (Figure 2.25B) unsurprisingly returned evidence of greater proportion of variance explained by inputted environmental factors, albeit still at low levels (5%). Of the components examined, RDA1 explained the most variance (1.9%), of which the factor SST makes the largest contribution. This was followed by RDA2 (1.3%). Only variances explained by RDA1, RDA2 and RDA3 were significant ($p<0.005$). No factors showed any evidence of collinearity.

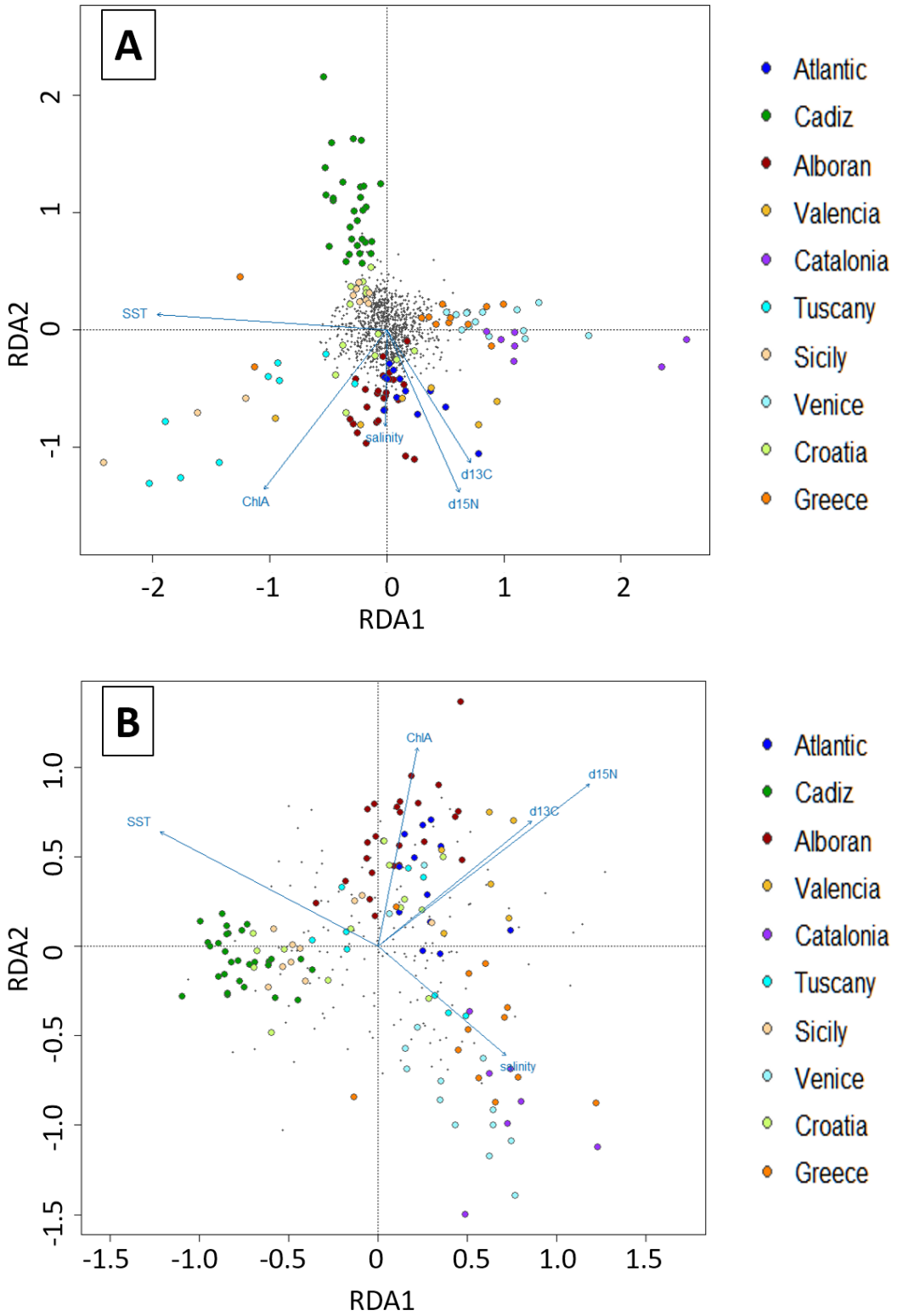

Figure 2.25: Redundancy Analysis (RDA) plots investigating the correlation between environmental variables and genetic variation. A) depicts neutral loci to examine pure population structure whereas B) uses only outlier loci to investigate local adaptation.

Analysis of environmental influences on population F_{ST} through *GESTE* v2.0 identified a mean population specific F_{ST} estimate of 0.351 (SD of 0.12). The constant only model was best supported (posterior probability = 0.777). This suggests that none of the modelled environmental factors (SST, salinity, chlorophyll A, $\delta^{13}C$ or $\delta^{15}N$) significantly influenced population-specific F_{ST}.

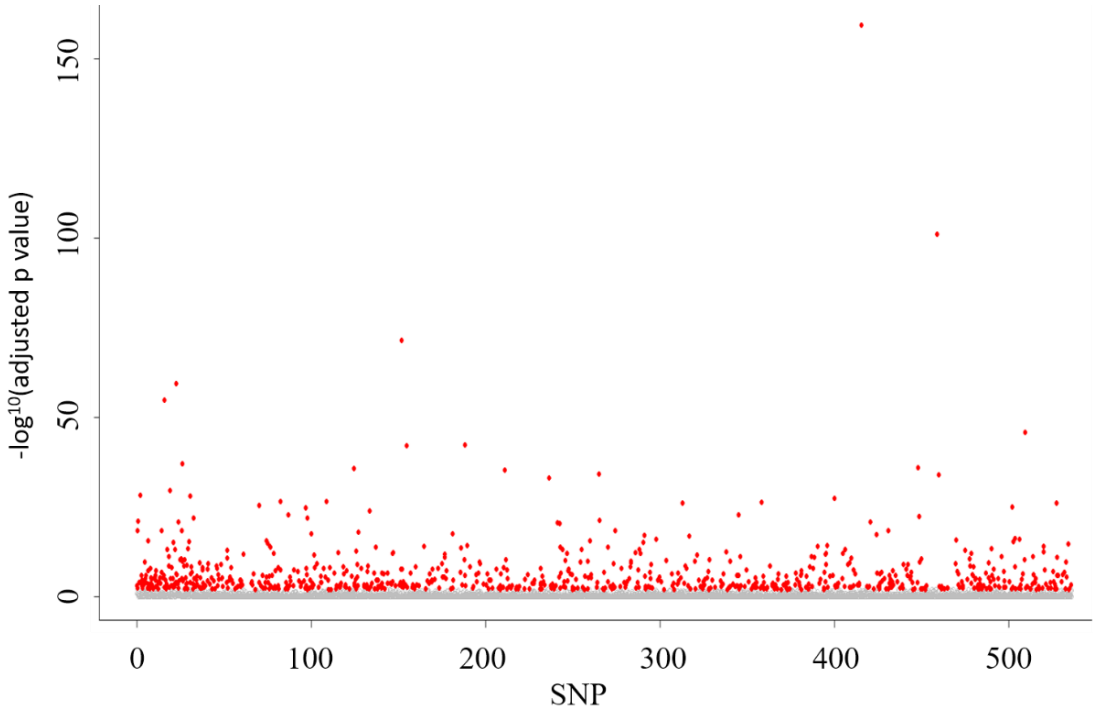

Figure 2.26: Manhattan plot of SNPs analysed for environmental association with LFMM. SNPs identified as having strong environmental association are coloured in red.

A total of 5104 SNPs were analysed for environmental association using LFMM and of these 815 SNPs were identified as outliers (Figure 2.26) with strong association to at least one environmental variable (SST, salinity and chlorophyll A). Unfortunately, even with Benjamini-Hochberg adjustment (Benjamini and Hochberg, 1995)of p-values with expected levels of FDR equal to q=5%, a histogram of p value frequencies revealed a u-shaped distribution (Figure 2.27) rather than the flat

distribution required. A flat histogram distribution indicates that p-values are drawn from a uniform distribution under the null hypothesis (Frichot and Francois, 2015). Therefore, the identified outlier SNPs cannot be utilised with confidence and no further progress was made in this analysis.

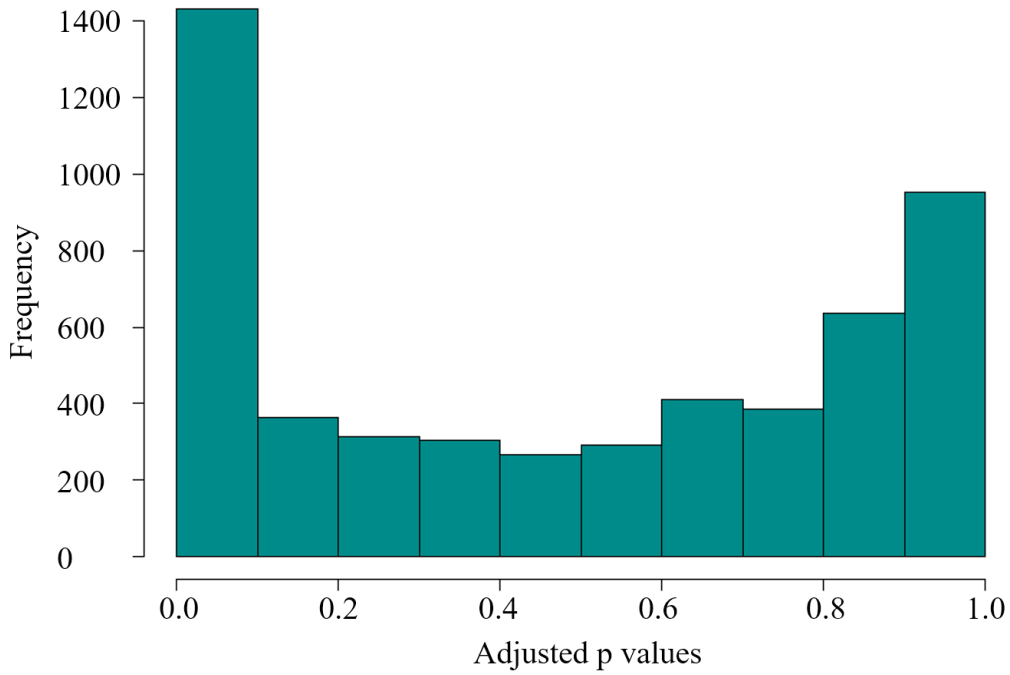

Figure 2.27: Frequency distribution of adjusted p-values for all SNPs (n=815) identified as being associated with at least one environmental variable in LFMM.

2.4 Discussion

This study set out to investigate how marine environmental features, principally oceanic fronts, could drive population structure formation in *Tursiops truncatus* and in so doing revealed a complex picture of structure where environmental features appear to be a key evolutionary driver.

Natoli *et al.* (2005) identified a clear East-West divergence in the population structure of *T. truncatus* in the Mediterranean Sea which coincided with the Siculo-Tunisian front (STF), something observed in other large marine predators (Boustany *et al*., 2008; Carlsson *et al*., 2004; Gaspari *et al*., 2007; Natoli *et al*., 2008). Excluding samples from Sicily, this study also found an East-West divergence in the Mediterranean. However, this divergence was aligned further west than that found by Natoli *et al*. (2005), possibly along the longitudinal line formed by Corsica and Sardinia, though paucity of samples in this region leaves that delineation somewhat ambiguous, much like the paucity of Sicilian samples in the Natoli *et al.* (2005) study may have led to their previous findings. Division along the Corsica-Sardinia line has been observed for other species in the Mediterranean (Davies *et al*., 2011; De Innocentiis *et al*., 2004; Montes *et al*., 2012). In their study of Gilthead Seabream *Sparus auratus* De Innocentiis *et al*. (2004) suggested the possibility that the population structure could be driven by larval retention in the Tyrrhenian circular gyre (Buffoni *et al*., 1997), something that could equally be applied to Albacore Tuna *Thunnus alalonga* as studied by Montes *et al*. (2012) and Davies *et al*. (2011). Although *T. truncatus* population structure could not be directly influenced via this mechanism the stable isotope results of this study clearly indicate differential feeding between the Tyrrhenian *T. truncatus* and those found along the Iberian coast (Valencia and Alborán); with the latter feeding at a higher trophic level. It is possible that prey

specialisation in Tyrrhenian *T. truncatus* may be restricting gene flow as the Tyrrhenian circular gyre limits prey distribution. Certainly both movements and distribution of *T. truncatus* has been shown to be strongly influenced by distribution of prey species (Hastie *et al*., 2004). Future studies of diet in *T. truncatus* of this region, as well as further samples of *T. truncatus* from Sardinia and Corsica for genetic study, would be useful for further investigation.

Like the STF, the Almería-Oran front (AOF) has been implicated in population structure formation in a number of marine predators (Bourret *et al*., 2007; Cimmaruta *et al*., 2005; Galarza *et al*., 2009; C. Schunter *et al*., 2011). This study revealed strong support (*Admixture* & *Barrier* analysis) for the AOF representing a population boundary in *T. truncatus*, something only lightly suggested in Natoli *et al*. (2005), with individuals found north of the Spanish city of Cartagena genetically differentiated from those found in the Alborán Sea. Although this study lacked samples from the Costa de Almería region and this geographical spacing necessitates a cautious interpretation - it does seem likely that the AOF is the relevant boundary to gene flow between *T. truncatus* found in the Alborán Sea and those further north off the coast of Valencia. As with the previously described Corsica-Sardinia line of divergence the stable isotope data in this study reveal clear signals of differential feeding between these populations, with *T. truncatus* from the Bay of Cádiz and Alborán Sea feeding at a higher trophic level than those off the coast of Valencia. In agreement with SIA data presented here, *T. truncatus* from the Bay of Cádiz are known to feed on large demersal fish species such as European Hake *Merluccius merluccius* and European Conger *Conger conger* (Giménez *et al*., 2017). Although larval retention by the AOF alone is unlikely to be enough to create a barrier to gene flow in potential prey species (Naciri *et al*., 1999), the AOF's strong temperature differential, caused by the meeting

of cold surface water from the Atlantic and warmer water flowing down from the Ibiza Channel, may be enough to influence species distribution and in so doing act as a potential mechanism for isolation of prey-specialist *T. truncatus* populations.

This pattern of oceanic frontal regions or steep environmental clines influencing population structure has been suggested for a number of cetacean taxa worldwide (Fontaine *et al*., 2007; Fullard *et al*., 2000; Kasuya *et al*., 1988; Mendez *et al*., 2011) including in *Tursiops* spp. (Bilgmann *et al*., 2007; Natoli *et al*., 2005). All of these studies implicate the oceanographic influence on prey distribution as being a likely key driver and the addition of stable isotope data in this study weakly supports this interpretation.

In addition to genetic divergence across the AOF, this study also found a broader geographic correlation between genetic population structure of *T. truncatus* and ocean water variables (SST and salinity). Specifically, salinity returned a significant result from Mantel tests for correlation with Nei's genetic D and during RDA significant test results were given where genetic variability was explained by SST (neutral loci) and salinity (outlier loci). Direct influence on *T. truncatus* physiology is unlikely to be causal mechanism here; this cosmopolitan species is known to tolerate a broad suite of environmental variables across their global range (Blanco *et al*., 2001; Olavarría *et al*., 2010), but environmental influence on distribution of prey species is again a possibility. Both salinity and SST are known to have a profound impact on the distribution of fish (Albert, 2007; Castillo, 1996; Sabatés *et al*., 2006), cephalopods (Fernández *et al*., 2011a; Lansdell and Young, 2007; Puerta *et al*., 2015) and crustaceans (Hall and Thatje, 2009; O'Hara and Poore, 2000), all of which are known to be prey species to a greater or lesser extent for different *T. truncatus* populations (Blanco *et al*., 2001; Giménez *et al*., 2017; Gladilina

and Gol'din, 2014; González *et al*., 1994; Santos *et al*., 2001). It should be taken in to account that the strong genetic divergence of Black Sea *T. truncatus* from other populations and the significantly lower salinity in the Black Sea likely has an influence on the Mantel test results in this study. However, the correlation between distribution of cetaceans and ocean environmental features, driven by the physiological limits of prey species, is well documented (Selzer and Payne, 1988; Tynan *et al*., 2005) and so it remains likely that prey distribution is a principal driver of genetic differentiation in *T. truncatus* in the Mediterranean, as has been suggested before for this species (Bilgmann *et al*., 2007; Fruet *et al*., 2014; Natoli *et al*., 2005; Sellas *et al*., 2005).

The Black Sea population of Bottlenose Dolphins have been identified as a genetically distinct population previously (Moura *et al*., 2020, 2013; Natoli *et al*., 2005), and it is now viewed as a subspecies of *T. truncatus* (*T. truncatus ponticus* (Viaud-Martinez *et al*., 2008)). This study concurred with this assessment; the Black Sea was the most genetically distinct population and consistently clustered together throughout analyses as well as producing the highest F_{ST} values. Whilst *T. truncatus* spp. are seen within the Istanbul Strait (also known as the Bosporus) (Bas *et al*., 2017), this body of water that separates the Black Sea from the Sea of Marmara is not only physically restricted (700m wide at its narrowest) but is also one of the busiest shipping lanes in the world with high levels of anthropogenic disturbance. As such, *T. truncatus* spp. encounter rates are significantly higher in the adjacent seas (Akkaya Baş *et al*., 2019) and the Istanbul Strait presents a strong physical barrier to gene flow that, alongside possible specialisation for prey species that may be geographically restricted by the lower salinity waters of the Black Sea, maintains genetic separation between the Black Sea and Mediterranean populations.

What emerges here is a clear pattern of population structure in *T. truncatus* being driven by physical parameters of the marine environment, either directly via physical restriction (the Istanbul Strait) or indirectly via prey distribution over scales both fine (ocean fronts) and large (environmental gradients). Within evolutionary biology the environmental influence on genetic differentiation in predator populations, particularly by impact on prey distribution, has been observed widely in both marine (Hoelzel, 2009; Olavarría *et al*., 2010) and terrestrial (Carmichael *et al*., 2001; Pilot *et al*., 2010) environments.

This study revealed a longitudinal separation between *T. truncatus* putative populations found in the northern Adriatic. Bottlenose dolphins found in the coastal waters of the Balkan states of Croatia and Slovenia demonstrated an admixture coefficient suggestive of descent from a differing ancestral population than those dolphins found off the north-western coast of Italy. Coastal *T. truncatus* in Balkan waters of the northern Adriatic are relatively well studied, thanks to the organisation Morigenos, and are known to exhibit fairly high site fidelity (Bearzi *et al*., 1997; Genov *et al*., 2009, 2008). Due to this ongoing monitoring it is known that very few individuals move between study sites, thus potentially limiting gene flow (Genov *et al*., 2009). This is not the first time that a longitudinal separation has been proposed for *T. truncatus* in the Adriatic, Gaspari *et al*. (2015a) also noticed a potential split between East and West Adriatic Bottlenose Dolphins. The Adriatic is characterised by measurable variability in habitat type and environmental conditions. The principal contemporary difference between the east and west northern Adriatic is salinity. The Italian side of the basin is heavily influenced by the river Po, reducing the salinity levels of the waters along the coast north of the estuary and for a considerable distance to the south (Russo and Artegiani, 1996). It is possible that local adaptation to this

slightly fresher environment, or specialisation in feeding on locally adapted prey, could be enough to present the genetic separation observed.

Since the LGM the Adriatic Sea has gradually pushed northwards as sea levels have risen, opening several new habitats in turn. Around 10,000 years ago the north western part of the Adriatic was a large barrier-lagoon estuary system, much like the favoured foraging grounds of *T. truncatus* in South Carolina (Gubbins, 2002; Pate and McFee, 2012), whilst the modern island-dominated coastal area of Croatia was all still dry land (Trincardi *et al*., 1996). The present Balkan coastline only became flooded later; thus, it is possible that this temporal succession of habitats, and environmental differences between them, could have provided the context for the initial formation of the population structure observed in this study through a series of founder events. Similarly, Gaspari *et al*. (2015b) proposed that a series of founder events could be responsible for the observed population structure of *T. truncatus* in the wider Eastern Mediterranean region. However, further factors must be responsible for contemporary maintenance of observed population structure in the Adriatic, with prey and/or habitat specialisation being a possible mechanism.

An interesting observation in this study was that *T. truncatus* from Sicily, Valencia and the Azores, although still showing clear inter-population differentiation (See F_{ST} results, Table 2.2), consistently clustered together during analyses (Figures 2.11-17). Sicilian *T. truncatus* have been shown to share acoustic characteristics in their vocalisations with their Macaronesian (Azores, Madeira and Canary Islands) counterparts (Papale *et al*., 2014) and there is greater acoustic similarity (in start, end and Delta frequency and number of inflection points) between these populations than any others that have been studied in the Mediterranean (La Manna *et al*., 2017). These studies refer specifically to narrowband, frequency-modulated signals that exhibit

clear contours of the fundamental frequencies (Caldwell *et al*., 1990). The vocalisations are predominantly expressed during observed social interactions and presumably for communication purposes (Steiner, 1981).

Vocalisations of *T. truncatus* have been categorised into two types: signature whistles and variant whistles (Caldwell *et al*., 1990). Signature whistles are unique to individuals, stereotypic and stable over time (May-Collado and Wartzok, 2008; Papale *et al*., 2014). It is thought that this type of signal is used for group cohesion, plays a dominant role in social interaction and can allow for individual recognition (Janik and Slater, 1998; Sayigh *et al*., 1999; Tyack, 1986). It is perhaps unsurprising that the sharing of acoustic characteristics would be a feature between populations with higher gene flow as multiple acoustic transmission methods have been observed for signature whistles, all of which incorporate social behaviours related to potential gene flow. All male groups and close male-male pair *T. truncatus* are known to be able to incorporate characteristics from each other's signal whistles in to their own repertoire, eventually converging on a common shared whistle used by all individuals in the alliance (Smolker and Pepper, 1999). Adoption of whistle characteristics is also documented in *T. truncatus* calves, both from their mothers (Sayigh *et al*., 1990) and from other community members, even ones with whom they encounter only occasionally (Fripp *et al*., 2005).

Beyond acoustics there are further similarities between the Sicilian and Macaronesia *T. truncatus* populations. Papale *et al.* (2017) conducted a study of social association patterns and site fidelity in the *T. truncatus* found off the south coast of Sicily and found that *T. truncatus* in this region had high social fluidity, with individuals associating in groups changing at relatively short timescales, and very low site fidelity (40% of dolphins sighted were only seen once and even those individuals

that were deemed ‘more resident’ left the study area for significant periods of time). This is in contrast to populations observed elsewhere in the Mediterranean (Bearzi *et al*., 2008; Benmessaoud *et al*., 2013; Blasi and Boitani, 2014; Díaz López and Shirai, 2008; Genov *et al*., 2008, 2008; Gnone *et al*., 2011; Pleslić *et al*., 2015) and much more akin to those seen in Macaronesia (Dinis, 2014; Silva *et al*., 2008).

In a study of *T. truncatus* of the Sicilian Channel Alessi *et al*. (2018) reported that over half (56%) of encounters took place whilst dolphins were engaged in feeding behind fishing vessels (as was observed during fieldwork for this study), similar to the high perceived level of fishery interaction reported off the coast of Valencia (Revuelta *et al*., 2018). In both these areas the main fishing fleets comprise pelagic trawl vessels fishing for small planktivorous fish, representative of lower trophic level feeding. Smaller planktivorous fish species are also reported to form an important part of the diet of *T. truncatus* found off the Azores (Clua and Grosvalet, 2001). This is in agreement with the stable isotope data from this study that supports a lower level of trophic feeding for *T. truncatus* in all three of these locations. It should be made clear here that this correlation more likely represents commonalities of feeding opportunities in a pelagic environment rather than a real driver of genetic differentiation between habitats.

These reported similarities between Sicilian *T. truncatus* and those found in Macaronesia, taken with the evidence of higher interpopulation connectivity provided by this study, suggests that these populations, with the possible additional inclusion of those *T. truncatus* found off the coast of Valencia (though these lack data to support acoustic or social structure similarities), could form a dispersed, and potentially offshore ecotype, metapopulation. This thesis lacks the data to be conclusive on this hypothesis but future studies, given increased sample availability, of population

structure, trophic ecology, bioacoustics and social structure may provide further evidence.

An interesting observation to come out of this study was that the Azores population appeared to have lower genetic diversity compared to the other *a priori* populations that were assessed, in particular the regions of East and West Italy. Given their trans-Macaronesian dispersal (Silva *et al*., 2008) this population is most probably of the offshore ecotype. It is therefore surprising that they appear to have lower genetic diversity as in other geographic regions, offshore populations have been shown to have greater genetic diversity than coastal populations (Goodwin *et al*., 1996; Segura *et al*., 2006; Sellas *et al*., 2005), indeed studies of mitochondrial DNA or microsatellites have suggested this to be true in the wider Atlantic region (Hoelzel *et al*., 1998; Parsons *et al*., 2002). It is possible that inference on genetic diversity for the Azores is limited due to this study only having samples from this particular locality when they are part of a geographically wider population.

Investigations of diet using $\delta^{13}C$ and $\delta^{15}N$ indicated a strong coastal and high trophic level for all *T. truncatus* individuals from the bay of Cádiz. The high $\delta^{13}C$ value for this group can likely be attributed to a strongly associated coastal lifestyle (see Michener and Kaufman, (2007)) and it could be argued that these individuals better fit the coastal ecotype rather than the pelagic ecotype as suggested by Nykänen *et al*. (2019). In either case, their enrichment of ^{15}N, and presumed feeding at a higher trophic level, fits with their known dietary preferences of large predatory fish species such as European Hake *Merluccius merluccius* and European Conger *Conger conger* (Giménez *et al*., 2017). These findings contrast strongly with those of the putative Azores-Sicily metapopulation which were depleted in ^{15}N and ^{13}C. The difference in pelagic vs coastal $\delta^{13}C$ has been used previously to differentiate between the two *T.*

truncatus ecotypes with offshore ecotypes usually being slightly depleted for ^{13}C (Barros *et al*., 2010), thus adding further evidence that Azorean, and possibly Sicilian, *T. truncatus* are of the offshore ecotype.

Investigations into the historical demographies of *T. truncatus* populations revealed a general trend of population decline over the past 100,000 years (with the exception of Atlantic and Greek populations which remained stable over this period). Some caution should be applied in the interpretation of these trends as many methods for estimating population historical demographics assume a model of a single panmictic population (Chikhi *et al*., 2018; Mazet *et al*., 2016), thus being influenced by admixture or violation of the panmixia assumption which can lead to inaccurate estimates of historical N_e (Grant, 2015). Effects of these violations can include false signals of population decline (Heller *et al*., 2013), though SFS methodologies, as used in this study, are known to be more resilient (Excoffier *et al*., 2013; Lohmueller *et al*., 2010). However, such long-term declines have been seen in other cetacean species such as Sperm Whales *Physeter macrocephalus* (Warren *et al*., 2017), Orca *Orcinus orca* (Moura *et al*., 2014) and several baleen whale species (Árnason *et al*., 2018; Kishida, 2017). These population declines have occurred over the past two million years and represent an as yet not fully understood phenomenon affecting a wide variety of cetacean taxa, adding confidence to the general trends observed in this study.

Although I can cautiously accept that a general decline in population sizes is likely, there still needs to be careful interpretation of individual population demographical features. Nearly all population declines displayed a distinct stepped pattern, suggestive of potential threshold tipping points resulting from environmental changes. However, apart from the Sicilian population (which showed dramatic population declines immediately following the last two glacial periods) the periods of

steepest decline do not correspond with any of the major geologic or environmental events investigated and it is possible that recent admixture may be influencing either the timing or magnitude of these events (Lohmueller *et al*., 2010). Presuming demographic features are real, the Sicilian population suffered a major population bottleneck around 1.5-1.6 million years ago, which was at the same time as a serious restriction in the Gibraltar Strait caused by extensive glaciation and subsequent fall in sea levels (Gibert *et al*., 2003). Given the connectivity with Atlantic populations that this study has identified, a reduction in gene flow between the two populations may be a potential causal factor in not only this bottleneck but also the two large population declines at 18k and 50k years ago which occurred immediately after the most recent two glacial periods.

An interesting observation from the historical demographies is that the population trends of Valencia, Alborán and Cádiz are nearly identical. This could suggest they have all been influenced by some environmental factor, or admixture event, that is specific to the Iberian Peninsula, irrespective of the fact that they retain distinct genetic separation as shown through the population structure investigations of this chapter. A final observation can be made on the human influence on *T. truncatus* populations. It has often been assumed that observed population declines in localities like the Black Sea are a result of human-led reductions in local anchovy stocks which are a key prey species for *T. truncatus ponticus* (Baird *et al*., 1993; Viaud-Martinez *et al., 2008)*, as well as direct hunting, pollution and fisheries bycatch. However, although these may well be factors, the data presented here suggest that these could just be accelerating factors for already downward population trend.

The primary aim of this study was to investigate the potential environmental drivers for the formation of population structure in *T. truncatus*, with particular

reference to the influence of oceanic fronts. Excluding *T. truncatus* from Valencia and Sicily, all remaining Mediterranean *T. truncatus* presented evidence of an east-west divergence (through Admixture and LEA). However, this divergence was aligned further west that that found by Natoli *et al*. (2005), possibly along the longitudinal line formed by Corsica and Sardinia, meaning that the STF is an unlikely barrier to gene flow in this species. By contrast, the AOF correlated with a strong genetic gradient, that is to say that *T. truncatus* individuals found north of the Spanish city of Cartagena were genetically differentiated from those found in the Alborán Sea. Although increased sample coverage in the Alborán-Valencia region may be useful to determine the point of delineation, it seems that the AOF is the likely line of divergence, with heterogenous prey distribution and prey specialisation in *T. truncatus* providing a potential mechanism. This provides evidence for frontal systems influencing the population structure of *T. truncatus* in the Mediterranean and so I accept my first hypothesis. The likely influence of oceanic fronts on prey resource distribution, alongside broader basin environmental gradients that may affect prey distribution and the differential prey specialism in *T. truncatus* supported by geographically dissimilar stable isotope data, means that I can also accept my second hypothesis.

The third hypothesis of this chapter proposed that one or more environmental variables would correlate with population structure for *T. truncatus* in the study area, suggesting that environment is a driver in the formation of this structure. During mantel tests genetic differentiation correlated with salinity, and both salinity and SST significantly explained some genetic variation during RDA. There was some differential specialisation in diet observed between populations and it is likely that prey species may be limited in their distribution by environmental conditions, thus

providing an indirect mechanism of influence. Following this my third and final hypothesis is also accepted.

2.5 Conclusion

Environment is likely a key driver in the formation of population structure in *T. truncatus* with environmental influence of prey distribution and *T. truncatus* populations having prey specialisms that act as the mechanism for the population structure formation. This occurs at geographic scales both acute (ocean fronts) and broad (environmental gradients).

Evidence is presented for an Atlantic-Sicily metapopulation, with the possible inclusion of Valencia, that have shared prey preferences, acoustic characteristics, low site fidelity and fluid social structure, suggesting the possibility that social structure and communication may also be a potential driver of genetic differentiation, although this requires further investigation.

Going forward it is recommended that future work seeks greater resolution around potential delineation zones, such as around the AOF and in the Corsica-Sardinia region. There also needs to be a concerted effort to seek additional samples from the north African coast for inclusion in future studies. Studies of acoustic characteristics and social structure of the Valencia population should be carried out to see if they share similarities with the proposed Atlantic-Sicily metapopulation.

Drivers of genetic population structure in the Yellowmouth Barracuda *Sphyraena viridensis*

3.1 Introduction

Characterisation of the drivers of population structure in marine predators requires comparison between taxa in order to isolate and identify key influencing factors, be they environmental, social or otherwise. Indeed, comparison of studies across taxa may reveal influencing factors which are specific or important to a given taxa and not others, potentially revealing multiple pathways for the formation of population structure.

3.1.1 Sphyraena viridensis

Sphyraena viridensis Cuvier 1829, also known as the Yellowmouth Barracuda or sometimes just Yellow Barracuda, is a predatory actinopterygian that is found in the Eastern North Atlantic and throughout the Mediterranean (Figure 3.1)(de Morais *et al*., 2015). Like all Sphyraenidae, *S. viridensis* has a long fusiform body with a streamline, pointed snout and pronounced underbite of the lower jaw. The colouration of *S. viridensis* generally follows a pattern of silver body with dark traverse barring running from the dorsal surface to past the lateral line and with areas of green or gold, particularly around the head and dorsal surfaces (Figure 3.2). Although considerably smaller than *T. truncatus* (the largest specimen of *S. viridensis* recorded in the literature measured 114.5cm Total Length (TL) (Barreiros *et al*., 2002), though this study includes numerous individuals larger than this.) it is understood that their dietary

preferences are remarkably similar. In the eastern Mediterranean adult TL tends to fall between 35-40cm (Allam *et al*., 2004), with considerably larger individuals seen in the Atlantic (Barreiros *et al.*, 2002; Fontes and Afonso, 2017). The individuals utilised in this study also reflected this dichotomous morphology.

3.1.2 Sphyraena viridensis vs Sphyraena sphyraena

No studies on ageing or growth patterns currently exist for *S. viridensis* and our current understanding of the biology and ecology of this species is extremely limited (Villegas-Hernández *et al*., 2014). This can be attributed, at least in part, to *S. viridensis* often being confused with the slightly larger but remarkably similar *Sphyraena sphyraena* or European Barracuda. *S. viridensis* can be identified from *S. sphyraena* on the basis of two subtle external features: 1) scales are absent from the preoperculum of *S. viridensis* whereas *S. sphyraena* has scales present on both the anterior and posterior margins of the preoperculum; and. 2) the dark traverse bars along the length of the body extend below the lateral line in *S. viridensis* whereas they do not reach the lateral line in *S. sphyraena*. They can also be distinguished based on otolith morphometrics (Bourehail *et al*., 2015).

There is also the potential for a third and also similar species within the same geographical area; Pastore (2009) proposed that *Sphyraena intermedia*, found in the Gulf of Taranto, be recognised as an intermediary of *S. viridensis* and *S. Sphyraena* based on body shape, otoliths, dentition and structure of the pyloric caeca. *S. viridensis* is recognised as being thermophilic, at least relative to its conspecific *S. Sphyraena*, and throughout various parts of the Mediterranean its numbers are growing, possibly

due to warming water temperatures in this area (de Morais *et al*., 2015; Villegas-Hernández *et al*., 2014).

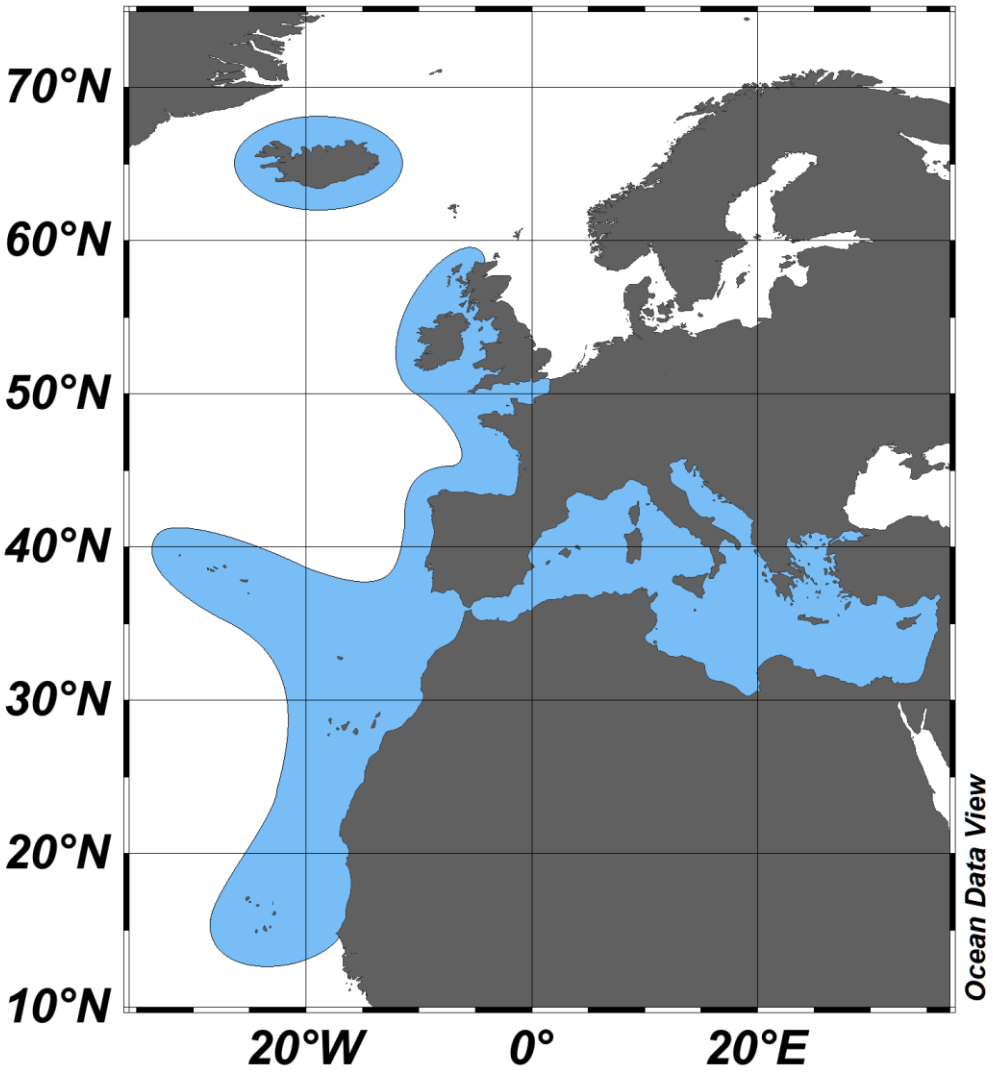

Figure 3.1. The known range of *Sphyraena viridensis*, the Yellowmouth Barracuda. Data from de Morais *et al.* (2015) and represent confirmed presence, actual range may be greater. Figure created with *Ocean Data View*.

3.1.3 S. viridensis in the family Sphyraenidae

There have been a number of attempts to establish an accurate phylogeny for the family Sphyraenidae (Daly-Engel *et al*., 2012; de Sylva, 1973; Milana *et al*., 2014; Santini *et al*., 2015; Soares *et al*., 2017). However, *S. viridensis* has only been attempted to be placed within this phylogeny on three occasions, firstly by Milana *et al*. (2014), secondly (using data from Milana *et al*. 2014) by Santini *et al*. (2015) and thirdly by Soares *et al*. (2017). As might be expected, all phylogenies place *S. viridensis* and *S. Sphyraena* monophyletically. Unfortunately, *S. intermedia* is rarely recognised and so has not been included in any phylogenies to date. Interestingly, this clade often has additions of other species that are not present in the Mediterranean. Both Santini *et al*. (2015) and Milana *et al*. (2014) include the Pacific Barracuda *Sphyraena argentea* which forms a monophyletic group with *S. viridensis* exclusive of *S. sphyraena.* Production of a timetree topology by Santini *et al.* (2015) suggests that *S. viridensis* separated from all other species by the end of the Miocene period (approximately 5.33 million years ago).

Figure 3.2: The Yellowmouth Barracuda, *Sphyraena viridensis*. Notice the absence of scales on the pre-operculum, distinguishing it from the similar *S. Sphyraena*. © Emily Cunningham.

3.1.4 Known genetic structure

Only a single study has examined the genetic structure of *Sphyraena viridensis* (Milana *et al*., 2014). This study examined three mtDNA regions (cytochrome oxidase I, cytochrome b and control region) in *S. viridensis* individuals found around the coast of Italy only. Samples were collected during summer 2012 by local fishermen and consisted of fin clips. Whilst cytochrome oxidase I and cytochrome b markers were principally used to confirm species identity (which they did successfully,

distinguishing *S. viridensis* from the similar *S. Sphyraena*), the mitochondrial (mtDNA) control region was utilised to elucidate population structure. The data from this study revealed 27 haplotypes which fell into two haplogroups (HgA and HgB) separated by nine mutational steps. The authors estimated that the split between the two groups most likely occurred sometime between 263,000 and 65,000 years ago based on the equation T=Da/2μ (Neethling *et al*., 2008) and a generally accepted teleost mutation rate of 3.6% per million years (Donaldson and Wilson, 1999).

Geographically the distribution of the observed haplogroups appeared non-random. Samples collected from within the Tyrrhenian Sea were dominated by HgA whereas all areas outside of this region were dominated by HgB. This differentiation may represent a population split along the line of the Siculo-Tunisian front but paucity of samples from the Sicilian coast prevents any hard conclusions from being drawn.

3.1.5 Trophic relationships and feeding

Sphyraena viridensis is known to feed predominantly on fish, with cephalopods and crustaceans making up a smaller proportion of their diet (Barreiros *et al*., 2002; Kalogirou *et al*., 2012), though *S. viridensis* in Egyptian waters of the Mediterranean have been found to feed exclusively on fish (Allam *et al*., 1999). Similarly, *T. truncatus* is known to also feed predominantly on fish with cephalopods and crustaceans as a supplement (Blanco *et al*., 2001). It could be hypothesised that, relative to *T. truncatus*, the prey choice of *S. viridensis* is gape-limited; forcing *S. viridensis* to preferentially select smaller baitfish which would likely give similar isotopic signals to the offshore *T. truncatus* found off the Azores and Sicily in Chapter 2.

3.1.6 Behaviour and reproductive ecology

Just a single study has examined the reproductive ecology of *S. viridensis*. Villegas-Hernández *et al*. (2014) examined 204 specimens collected over one calendar year in the Gulf of Lyon (north western Mediterranean) and found that females were capable of spawning for a two-month period from mid-May to mid-July, but with a peak in mid-June. The authors note an interesting correlation between seasonal patterns of landings and mean monthly sea surface temperatures, both of which peaked in July-August, something also observed in the Azores (Fontes and Afonso, 2017). Being water column spawners, *S. viridensis* larval dispersal is likely strongly linked to ocean currents and this may be reflected in their population structure, though preliminary and geographically limited studies have shown larval distribution to be independent of habitat (Blasi et al., 2013).

S. viridensis is a crepuscular species, being most active in hunting behaviour immediately pre-sunrise and post-sunset (Merciai *et al*., 2020). Hunting behaviour is typically solitary but during the day it is known to sometimes associate in large schools (Barreiros *et al*., 2002)(see Figure 3.4), presumably for increased protection from predators. *S. viridensis* is assumed to have no complex social structure like other Sphyraenidae; if one should exist it is highly unlikely to be comparable to that of *T. truncatus*.

3.1.7 Aims and hypotheses

This study will provide the first investigation into the population structure of *Sphyraena viridensis* across a substantial proportion of its range. Furthermore, it will provide the first examination of trophic structure in this species through stable isotope

analysis, with genetic structure data then being integrated with both stable isotope and environmental data to investigate the drivers of population structure formation.

Towards these aims this chapter will examine and test the following hypotheses:

Hypothesis 1:

H1: There is detectable population structure in *Sphyraena viridensis* across the geographic scope of this study.

Hypothesis 2:

H1: There are observable differences in diet between populations of *Sphyraena viridensis* across the study area examined.

Hypothesis 3:

H1: Observable population structure of *Sphyraena viridensis* in the Mediterranean and eastern Atlantic is correlated with one or more environmental variables that likely, either directly or indirectly, drive population structure formation.

3.2 Methodology

3.2.1 Sample collection

Various potential collaborators and partners around the Mediterranean Sea and eastern Atlantic were approached via email with a view to obtaining tissue samples of *Sphyraena viridensis*. A total of 40 samples of *S. viridensis* individuals were donated by the authors of Milana *et al*. (2014). These samples consisted of fin clips collected from specimens caught by local fishermen around the Italian coast. Further samples were donated by Dr. Joan Moranta of *Centro Oceanográfico de Baleares* (n = 6) which were collected by fishing vessels operating around the Balearic Islands and consisted of both fin clips (typically 3-4cm, taken from the dorsal edge of the caudal fin) and white muscle tissue (taken from the dorsal flank, usually just below the dorsal fin).

Following calls put out via the online Italian forums www.naturamediterraneo.com and www.pescanetwork.it four samples were donated from amateur fishermen located on Ponza Island (n = 3, white muscle tissue) and in Palermo, Sicily (n = 1, fin clip and white muscle tissue). Further samples (n = 5) were donated by Dr. Mireille Harmelin-Vivien of the *Institut Méditerranéen d'océanologie* and consisted of lyophilized muscle tissue left over from Stable Isotope research conducted as part of the doctoral thesis of Pierre Cresson. 14 samples were donated by Dr. Alberto Brito of the *Universidad de La Laguna* and consisted of white muscle tissue and were collected by local fishermen around Tenerife. Further samples (n=29, including six from fieldwork detailed below) were collected by Prof. João Pedro Barreiros of the *Universidade dos Açores* via spearfishing and donated to this research. Two additional samples were obtained from a supermarket fish counter on Terceira, Azores during fieldwork on the island. A total of 103 samples were available for use in this study.

3.2.2 Spearfishing

During the period 25-30th September 2017 sampling was conducted around the island of Terceira, Azores, using Apnea Spearfishing technique. The expedition team (Daniel Moore and Emily Cunningham) was hosted by Prof. João Pedro Barreiros of the *Universidade dos Açores* and supported by Centre for Ecology, Evolution and Environmental Changes (CE3C) (University of the Azores - Faculty of Agrarian and Environmental Sciences) through the use of their boat and campus facilities. Spearfishing equipment, including guns and wetsuits, were provided by Picasso (http://www.picasso.pt/) who sponsored Prof. João Pedro Barreiros. Spearfishing took place at 3 locations (North Cabras Islets (NCI), Fradhinos Islets (FI) and Porto Judeu (PJ)) on the South coast of Terceira (Figure 3.3). North Cabras Islets and Fradhinos were accessed via boat whereas Porto Judeu was a shore entry.

The bathymetry surrounding Fradhinos Islets is extremely deep, plunging to 400m depth within 100m horizontal distance from the islets. Currents around this site were strong and *S. viridenis* was observed in a single large school thought to exceed 200 individuals (Figure 3.4). Targeting large schools when spearfishing is difficult due to coordinated group evasive movements, however two individuals were successfully speared. Both North Ilhéus das Cabras and Porto Judeu were shallower (typically 10-20m depth), rocky reef environments. In both sites, currents were minimal and *S. viridensis* were observed individually or in pairs. One individual was speared at Porto Judeu but unfortunately no individuals were caught at North Ilhéus das Cabras.

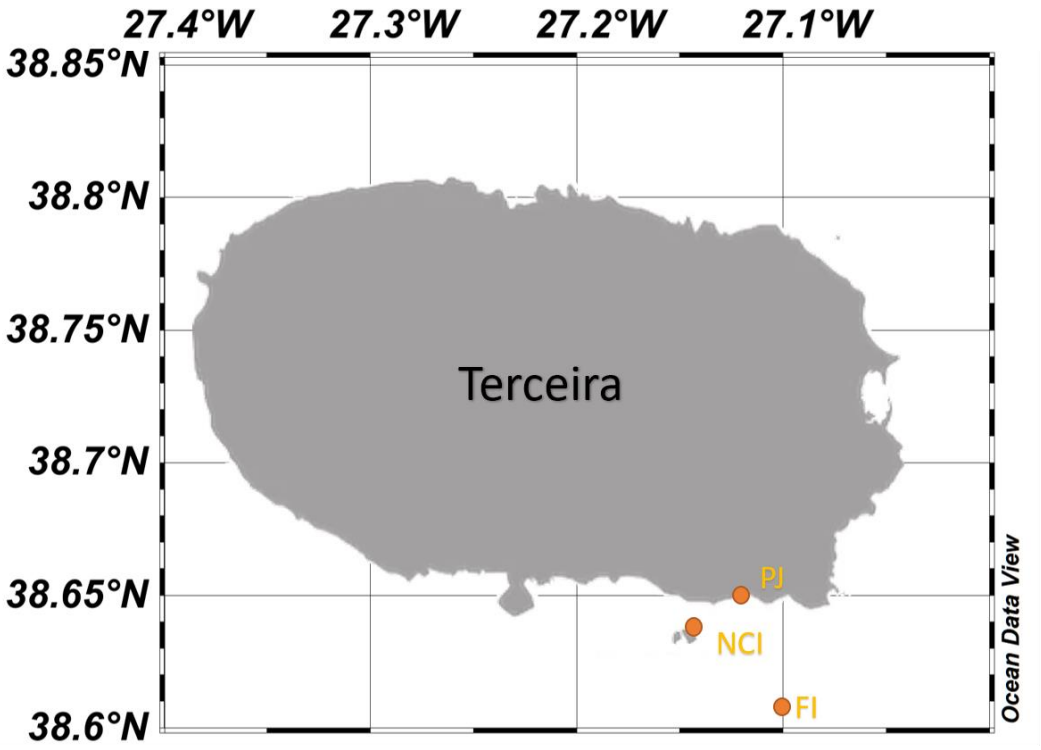

Figure 3.3: The Azorean island of Terceira and the collection sites that were targeted for spearfishing. PJ = Porto Judeu, NCI = North Cabras Islets and FI = Fradhinos Islets.

Figure 3.4: A large school of *Sphyraena viridensis* seen swimming off Fradhinos Islets, Terceira during September 2017. Estimated group size is greater than 100 individuals. © Daniel Moore

Collected *S. viridensis* were transported back to shore before being processed for samples, typically within 1-2 hours. Processing consisted of taking a total length (TL) measurement and then extracting a roughly 1x1x2cm piece of white muscle tissue from the dorsal flank just below the dorsal fin using a scalpel and dissecting scissors (Figure 3.5a) as well as a roughly 1x3cm fin clip from the dorsal margin of the caudal fin using bow scissors (Figure 3.5b). Tissue samples were stored in DMSO in 1.5ml Eppendorfs and frozen at -20°C before transportation back to Durham University. Final sample distribution available for use in this study is illustrated in Figure 3.6.

No permits were required for conducting this fieldwork but it was undertaken with full support of the *Universidade dos Açores* and appropriate field risk assessments were undertaken.

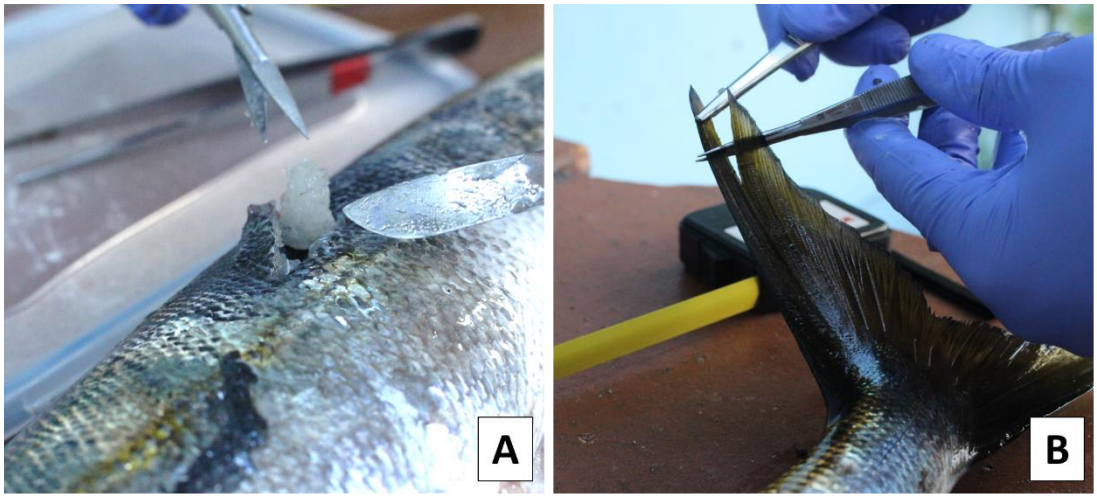

Figure 3.5: White muscle tissue was extracted from the dorsal flank of each *S. viridensis* individual (A) and fin clips were taken from the posterior dorsal region of the caudal fin (B). © Emily Cunningham

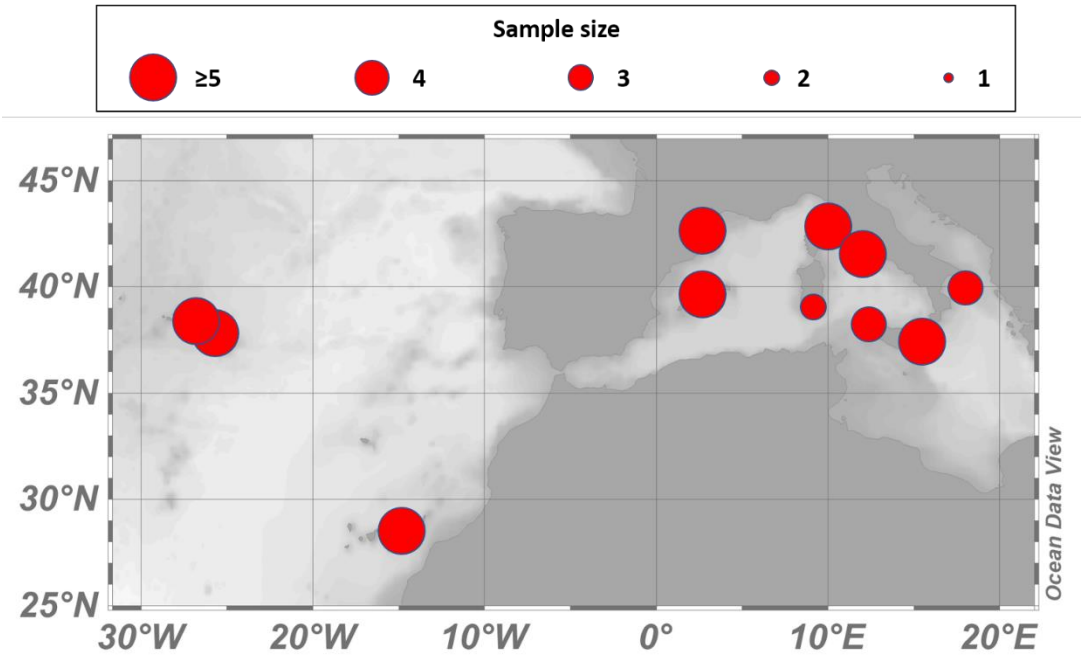

Figure 3.6: Geographic distribution of *Sphyraena viridensis* samples available for use in this study

3.2.3 DNA extraction

A standard phenol-chloroform extraction protocol was used to isolate *Sphyraena viridensis* DNA, largely following Hoelzel (1998) but see Chapter 2 for slight alterations. Preference was given to fin clippings when available, in which case a piece typically 0.5x0.5cm was used. If a fin clip was not available then 100mg of white muscle tissue (typically 0.5x0.5x0.5cm or smaller) was utilised.

Concentration of DNA extractions was evaluated using a Qubit 2.0 fluorometer (Invitrogen). Typically, at least two independent readings would be made of each extraction to ensure confidence of the measurement. DNA extraction quality was assessed using a Nanodrop spectrophotometer (Thermo Scientific). In a number of cases DNA concentration for *S. viridensis* extractions were found to be relatively low (<10ng/µl) and in this case fresh extractions were conducted and resuspended in the original extraction.

3.2.4 Library preparation

This chapter intended to utilise two pathways to detect genetic population structure. The first was selected as a traditional and resilient technique, namely amplification of mitochondrial DNA control region. The second sought to develop a new and ambitious but potentially rewarding method that aims to produce a multiplexed microsatellite bait capture library with potentially hundreds of microsatellite markers. Ultimately the second approach proved unsuccessful but there are lessons learned and methodological advances made so this information has been included in Appendix IV.

3.2.5 Mitochondrial DNA amplification

Mitochondrial sequences from samples utilised in Milana *et al.* (2014) were downloaded from GenBank (Accession numbers KJ396641-KJ396670, n=40). To add to these data, amplification of the mitochondrial DNA control region (Figure 3.7) was attempted from all additional samples (n=64) collected for this study. It was hoped to use only those primers published by Milana *et al*. (2014) but trials dictated the necessity for the design of additional primers for this study to be used in combination with those previously published for this species. All successful primer combinations for each sample are outlined in Table 3.2. The first primer (forward), L19 (5'-CCA CTA GCT CCC AAA GCT A-3'), is located in the proline tRI4A gene and was designed by Bernatchez *et al*. (1992) based on homologies seen among already published sequences from fish (Buroker *et al*., 1990; Johansen *et al*., 1990; Meyer *et al*., 1990). The second (reverse) primer, K-Rev (5'-CAG GAC CAA GCT TTT GTG CTT ACG-3') was designed by Milana *et al*. (2014). As this combination did not seem to work for all samples four additional primers were designed for use in this study

using published *Sphyraena* spp. mitochondrial control region data and the alignment software Clustal X (Thompson *et al*., 1997). Primers are defined in Table 3.1 but in brief these primers were F1 (5'- TTA GCA TTA GTA GCT CAG-3'), F2 (5'- TTT AGT CGT CGG AGG TTA-3'), R1 (5'- GAT AGT AAA GTC AGG ACC-3') and R2 (5'- CCA TCC TAA CAT CTT CAG-3'). Designations 'F' and 'R' in primer names indicate Forward and Reverse respectively.

A Polymerase Chain Reaction (PCR) was set up in a total volume of 50µl, containing 36.25µl pure H_2O, 5µl 10X Standard Taq Buffer (New England Biosystems), 1µl 10mM dNTPs, 2.5µl 10µM L19 forward primer, 2.5µl 10µM K-Rev reverse primer, 1.25µl 25µM $MgCl_2$, 0.5µl Taq DNA Polymerase (5,000 U/ml, New England Biosystems) and 1µl 10-100ng/µl template. PCR was run in an Applied Biosystems Veriti 96 well thermal cycler under the following conditions: initial denaturation of 5 minutes at 95°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 60 seconds at 72°C and a final extension of 10 minutes at 72°C.

PCR products were cleaned using Exo-SAP-IT (Affymetrix) whereby 2µl of Exo-SAP-IT were added for every 5µl of PCR product and held at 37°C for 15 minutes in a thermal cycler followed by an Exo-SAP deactivation step of 15 minutes at 80°C. Final concentration was checked using a Qubit 2.0 fluorometer (Invitrogen).

Table 3.1: Primer sequences used to amplify mitochondrial DNA sequences of *Sphyraena viridensis*.

Primer Name	Sequence
L19	5'-CCA CTA GCT CCC AAA GCT A-3'
Krev	5'-CAG GAC CAA GCT TTT GTG CTT ACG-3'
F1	5'- TTA GCA TTA GTA GCT CAG-3'
R1	5'- GAT AGT AAA GTC AGG ACC-3'
F2	5'- TTT AGT CGT CGG AGG TTA-3'
R2	5'- CCA TCC TAA CAT CTT CAG-3'

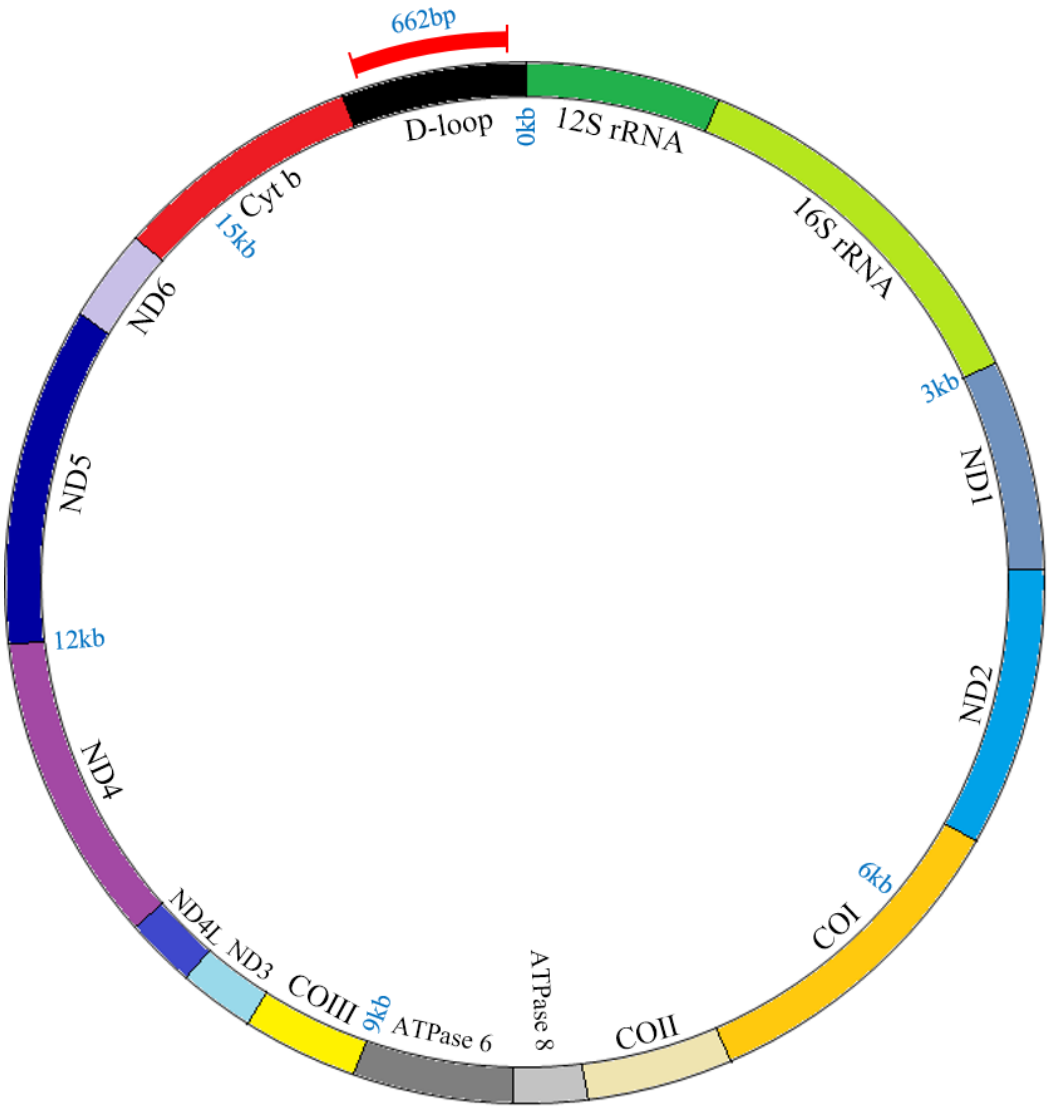

Figure 3.7: Mitogenome map from *Sphyraena jello* (based on Lv *et al*. (2016)) showing the section of mtDNA being amplified in this study (highlighted in red above the D-loop) for *S. viridensis*.

3.2.6 Sequencing

Cleaned mitochondrial control region PCR products were submitted for sequencing at DBS Genomics, Durham University and run on an Applied Biosystems 3730 capillary DNA Analyzer. Sequencing was carried out in a single direction only.

Table 3.2: Successful primer combinations used to amplify mitochondrial DNA sequences of *Sphyraena viridensis.*

Sample	Primer Pair	Sample	Primer Pair	Sample	Primer Pair
Sv41	L19+Krev	Sv62	L19+R2	Sv85	F1+R2
Sv42	L19+Krev	Sv63	L19+R2	Sv86	F1+R1
Sv43	L19+Krev	Sv64	L19+R2	Sv87	F1+R1
Sv46	L19+R2	Sv66	F1+R2	Sv92	F1+R2
Sv47	L19+Krev	Sv68	L19+R2	Sv93	F1+R1
Sv48	L19+R2	Sv71	L19+R2	Sv94	F1+R1
Sv49	L19+R2	Sv73	L19+R2	Sv95	F1+R1
Sv50	L19+R2	Sv75	L19+R2	Sv96	F1+R1
Sv51	F1+R1	Sv76	F1+R2	Sv99	F1+R1
Sv53	L19+R2	Sv78	L19+R2	Sv101	F1+R1
Sv57	F1+R1	Sv79	F1+R2	Sv102	F1+R1
Sv58	F1+R1	Sv80	F1+R2	Sv103	F1+R1
Sv59	L19+R2	Sv81	F1+R2	Sv104	F1+R1
Sv60	F1+R1	Sv84	F1+R2		

3.2.7 Stable Isotope Analysis

All tissue samples were kept frozen at -20°C prior to preparation for Stable Isotope Analysis (SIA). Tissue samples from *Sphyraena viridensis* consisted of both white muscle tissue and a caudal fin clip (See Appendix IV for details) although both tissue types were not available for every individual. Where both tissue types were present preference was given to fin clips for SIA. Preparation of samples and mass spectrometry details were the same as followed for *Tursiops truncatus* – please see sections 2.2.7-8 for further details.

3.2.9 Analyses

Mitochondrial DNA (mtDNA) control region raw chromatographs from successfully amplified samples were edited and aligned using *CodonCode Aligner* 9.0.1 (CodonCode Corporation). These sequences were combined with those downloaded from Milana *et al.* (2014) using *MUSCLE* (Madeira *et al*., 2019) with resulting alignments exported in NEXUS and FASTA formats. Population pairwise F_{ST} values were calculated using *Arlequin* v3.5 (Excoffier and Lischer, 2010) with Bonferroni correction. Aligned sequence data were loaded into *R* using the package *ape* (Paradis and Schliep, 2019) and Φst was calculated using the *PairPhiST* function in the *R* package *Haplotypes* (Aktas, 2015).

A phylogenetic tree using the Neighbour Joining method was created in *R* using the package *Phangorn* (Schliep, 2011) following the performance of a likelihood ratio test to establish the most appropriate model of nucleotide evolution. A second tree was created following a Maximum Likelihood method, also in *R* using the package *Phangorn*, but rooted with the homologous sequence from the Great Barracuda, *Sphyraena barracuda* downloaded from Genbank (Accession number: NC_022484.1). This tree was optimised with the function *optim.pml*, bootstrapped with 1000 iterations and node support set at 50%.

Production of an absolute pairwise difference network using statistical parsimony was produced using the function *parsimnet* in the *R* package *haplotypes*. PCoA was conducted using the function *glPca* from package *Adegenet* (Jombart, 2008) following conversion of data to genlight format using function *gi2gl* from package *dartR*.

Stable isotope data was manipulated in *Microsoft Excel* and isoscape plots were produced using *Ocean Data View* (Schlitzer, 2018). Statistical tests of variability in isotope values between geographic areas were conducted in *Minitab* v14.

Mantel tests were performed to test correlation between a genetic distance matrix (Jost's Pairwise D (Jost, 2008), calculated via *FinePop* in *R*) and distance matrices of environmental variables (SST, Salinity and stable isotope values for $\delta^{13}C$ and $\delta^{15}N$). Sea Surface Temperature (SST) comes from CNR-Med satellite data (Nardelli *et al*., 2013) and salinity data are derived from the Copernicus Marine Environment Monitoring Service (von Schuckmann *et al*., 2016). Tests were performed using the package *Vegan* in *R* and set with 999 permutations and a Pearson model. Redundancy Analysis (RDA) was conducted using the packages *Vegan* and *psych* in *R*. RDAs were ran with geographic distance, SST, salinity, $\delta^{13}C$ and $\delta^{15}N$ as explanatory variables and genetic distance (Jost's Pairwise D) as the response variable. Basic statistical testing was conducted using *Minitab* v1.4.

3.3 Results

Either muscle or fin clip samples from a total of 103 *Sphyraena viridensis* individuals were collected for this study (See Appendix IV). Following DNA extraction, samples were tested for concentration, quality and contamination. DNA extractions from only 71 individuals were deemed suitable for further study due to low yield or quality issues with the remaining 32. Analysis of mtDNA Control region (CR) was conducted on 40 individuals whose sequences were downloaded from GenBank (from Milana *et al.* (2014)) plus the additional 33 successful extractions completed for this study meaning representation was made covering all geographic areas of interest. The attempt to analyse all 71 successful extractions with the microsatellite bait-capture protocol was ultimately unsuccessful (see Appendix IV). Finally, stable isotope analyses were conducted on all 103 individuals.

3.3.1 Mitochondrial DNA analyses

Alignment of mtDNA control region sequences resulted in a 649bp consensus sequence from 73 individuals. The consensus sequence consisted of 34.7% Guanine/Cytosine bases with an overall respective breakdown of 211 bases Adenine, 117 bases Cytosine, 108 bases Guanine and 193 bases Thymine. Across the consensus sequence there were 63 polymorphic sites and a total of 52 unique haplotypes.

Population genetic differentiation was investigated through examination of F_{ST} and Φ_{ST} values (Table 3.3). A cautious approach to interpretation should be taken with these values as some population groups have low sample sizes (<10) which is known to produce inflated values (see Holsinger and Weir (2009) for a review). However, whilst still accepting low reliability of values general trends can be

observed. F_{ST} values revealed high levels of differentiation between the Tyrrhenian and four other tested groups. Three of these groups had low sample sizes, however the third group (Azores) did not and a more confident interpretation of this can be taken, thus showing a high level of divergence between Atlantic and Mediterranean *S. viridensis*, something that is also supported by a significant and high Φ_{ST} between the Tyrrhenian and Azores groups. Inspection of Φ_{ST} supports high levels of divergence between the two Atlantic populations and several other groups as well as between each other. However, caution must be stressed again to avoid overinterpretation of these values. In an attempt to overcome low sample sizes all samples east of Italy were pooled and tested for the same metrics with the already larger sample sets of the Tyrrhenian and the Azores (Table 3.4). This revealed strong differentiation either side of Italy but relatively low but still detectable differentiation between east Italy and the Azores.

Table 3.3: Population pairwise F_{ST} values (below the diagonal) calculated using Arlequin v3.5 and Φ_{ST} (above the diagonal) calculated using the *R* package *Haplotypes* based on mtDNA. Values in bold are significant at p<0.05. Populations with n<10 are indicated with *.

	Tyrrhenian	Ionian*	Sicily*	Sardinia*	Adriatic*	Mallorca*	Azores	Canaries*
Tyrrhenian		**0.392**	**0.238**	0.277	**0.286**	0.000	**0.374**	0.0578
Ionian*	**0.392**		0.020	0.143	0.000	0.222	**0.132**	**0.303**
Sicily*	**0.238**	0.020		0.000	0.000	0.067	0.024	**0.195**
Sardinia*	0.277	0.143	-0.175		0.000	0.000	0.000	0.000
Adriatic*	**0.286**	-0.099	-0.015	-0.151		**0.110**	**0.099**	**0.263**
Mallorca*	-0.006	**0.216**	0.067	-0.138	0.109		**0.215**	0.000
Azores	**0.368**	0.119	0.018	-0.239	**0.092**	**0.206**		**0.336**
Canaries*	0.059	0.178	**0.068**	-0238	0.121	-0.049	**0.184**	

Table 3.4: Population pairwise F_{ST} values (below the diagonal) and Φ_{ST} (above the diagonal) for broad geographical areas based on mtDNA. Values in bold are significant at p<0.05.

	Tyrrhenian (n = 24)	East Italy (n = 17)	Azores (n = 17)
Tyrrhenian		**0.320**	**0.374**
Ionian	**0.320**		**0.097**
Azores	**0.368**	**0.092**	

PCoA analysis (Figure 3.8) revealed two clear clusters that did not correlate with geographic origin and with PC1 explaining nearly 60% of variation. There is a general trend however for cluster divergence between Tyrrhenian and Azorean individuals, with a slight possible bias for Ionian samples to cluster with those of the Azores.

First investigations into phylogenetic relationships were examined using a non-rooted neighbour joining tree (Figure 3.9). This early inspection reveals that the same cluster of individuals, largely from the Tyrrhenian, that was identified on the left of the plotted PCoA (Figure 3.8) forming a clade with a significant evolutionary distance from all other individuals. This was investigated further with a more robust maximum likelihood (ML) tree (Figure 3.10). Maximum likelihood methods allow for more robust interpretations due to taking into account all sequence data, not just distance matrices used for neighbour-joining methods. Greater reliability was added through rooting with the homologous sequence of the Great Barracuda *Sphyraena barracuda* and 1000 bootstraps. The ML tree reveals a clear two-clade structure.

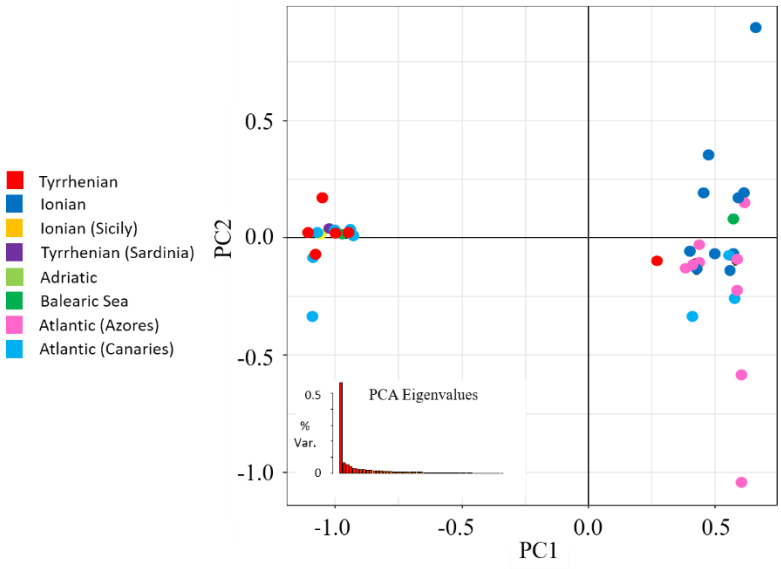

Figure 3.8: PCoA for *S. viridensis* mtDNA revealing two clear haplogroups. Individuals are coloured by geographic origin.

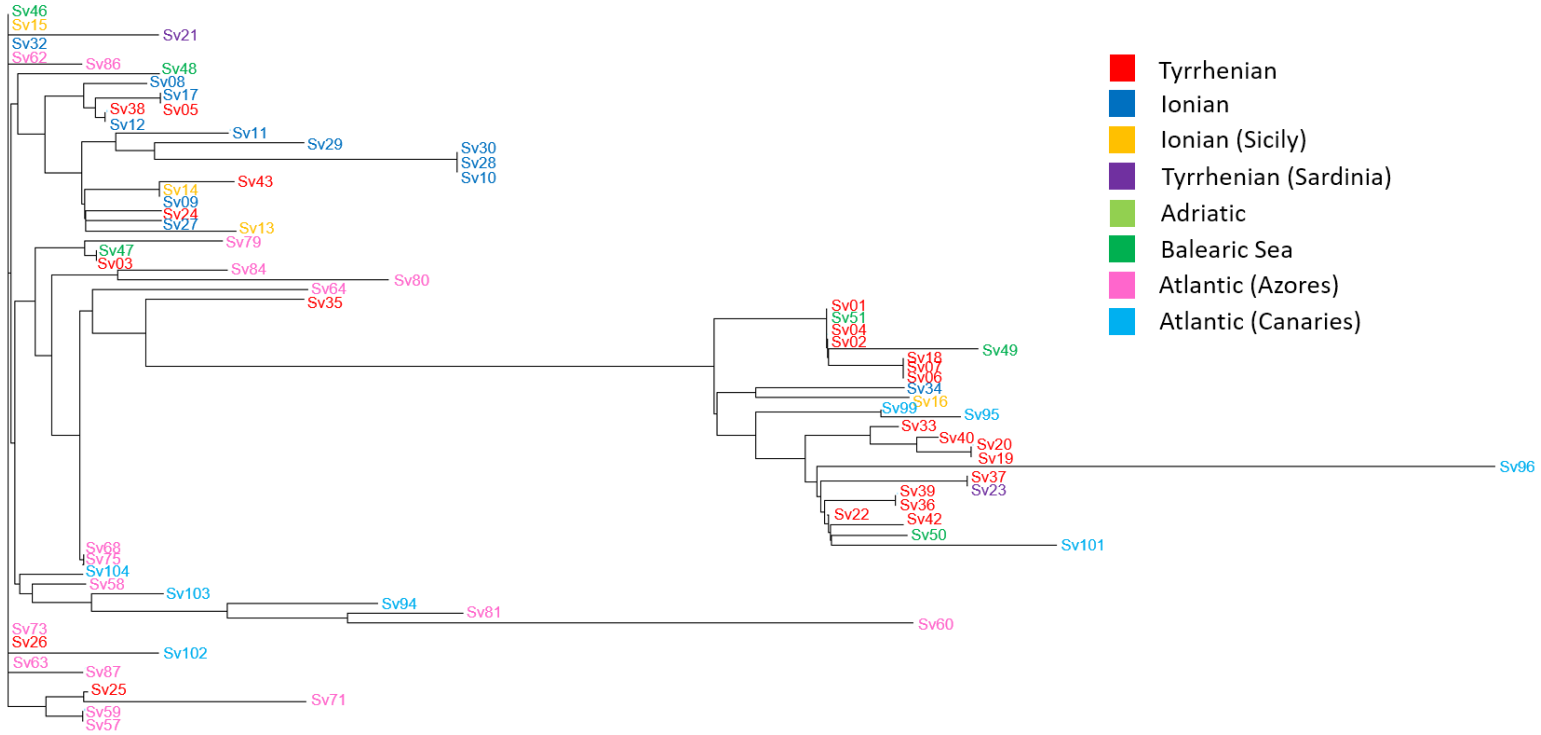

Figure 3.9: Unrooted Neighbour joining tree, without bootstrapping, for *S. viridensis* mtDNA data. Sample names are coloured by geographic origin.

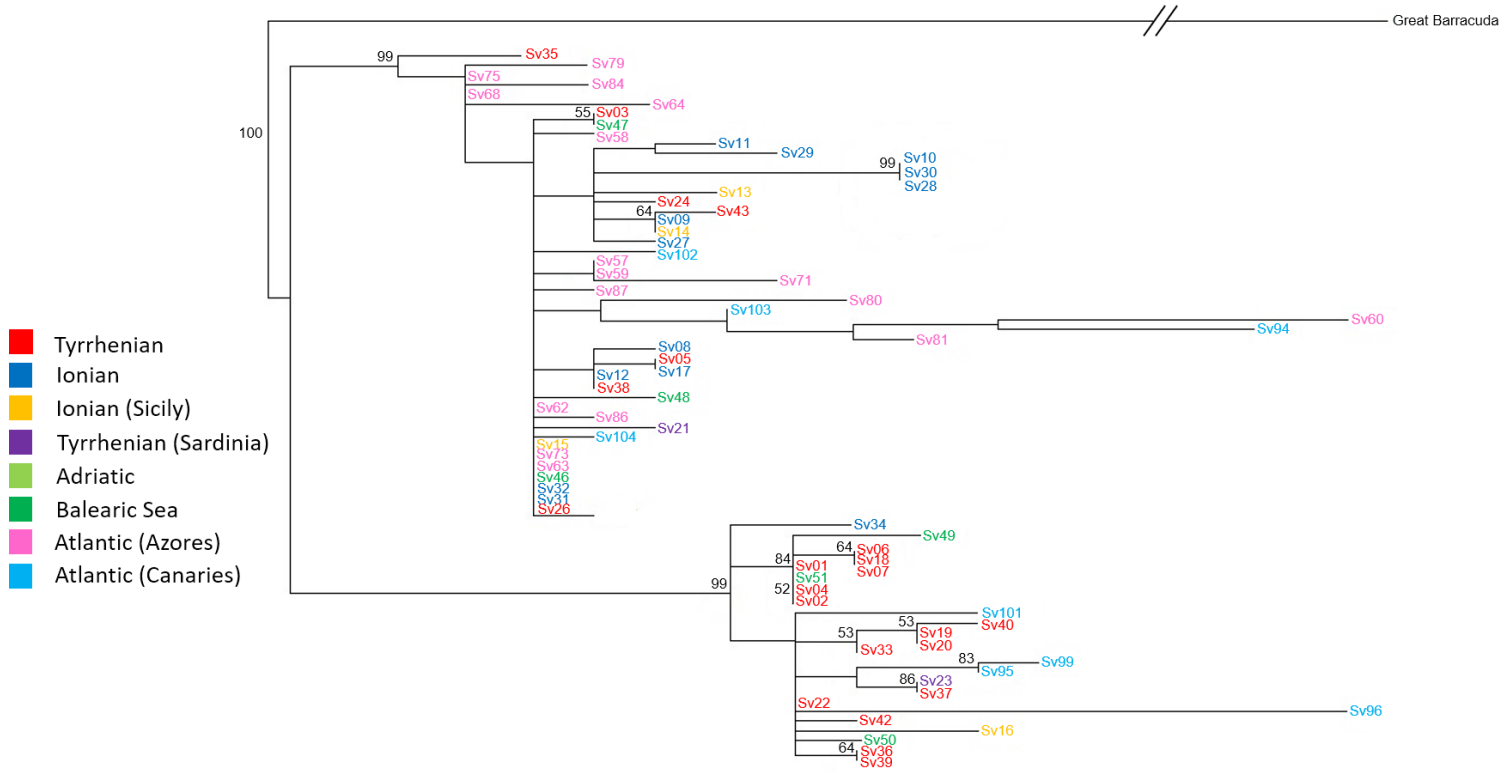

Figure 3.10: Maximum Likelihood tree of *S. viridensis* mtDNA data, rooted with the homologous sequence of Great Barracuda *Sphyraena barracuda* and with 1000 bootstraps. Nodes with greater than 50% bootstrap support are shown by black numbers.

To investigate this two-clade structure further, a parsimonious haplotype network was created using the *parsimnet* function of the *R* package *haplotypes* (Figure 3.11). This haplotype network presents the clearest evidence of the presence of two haplogroups present in *S. viridensis*, something previously presented in Milana *et al.* (2014). Figure 3.11 shows the haplogroups labelled HgA and HgB, as in Milana *et al.* (2014) and that all additional samples collected for this study readily sit in either of the two haplogroups. There is significant unequal distribution of sample geographic origin between the haplogroups (Chi-squared test, X2 = 239.9, P<0.001).

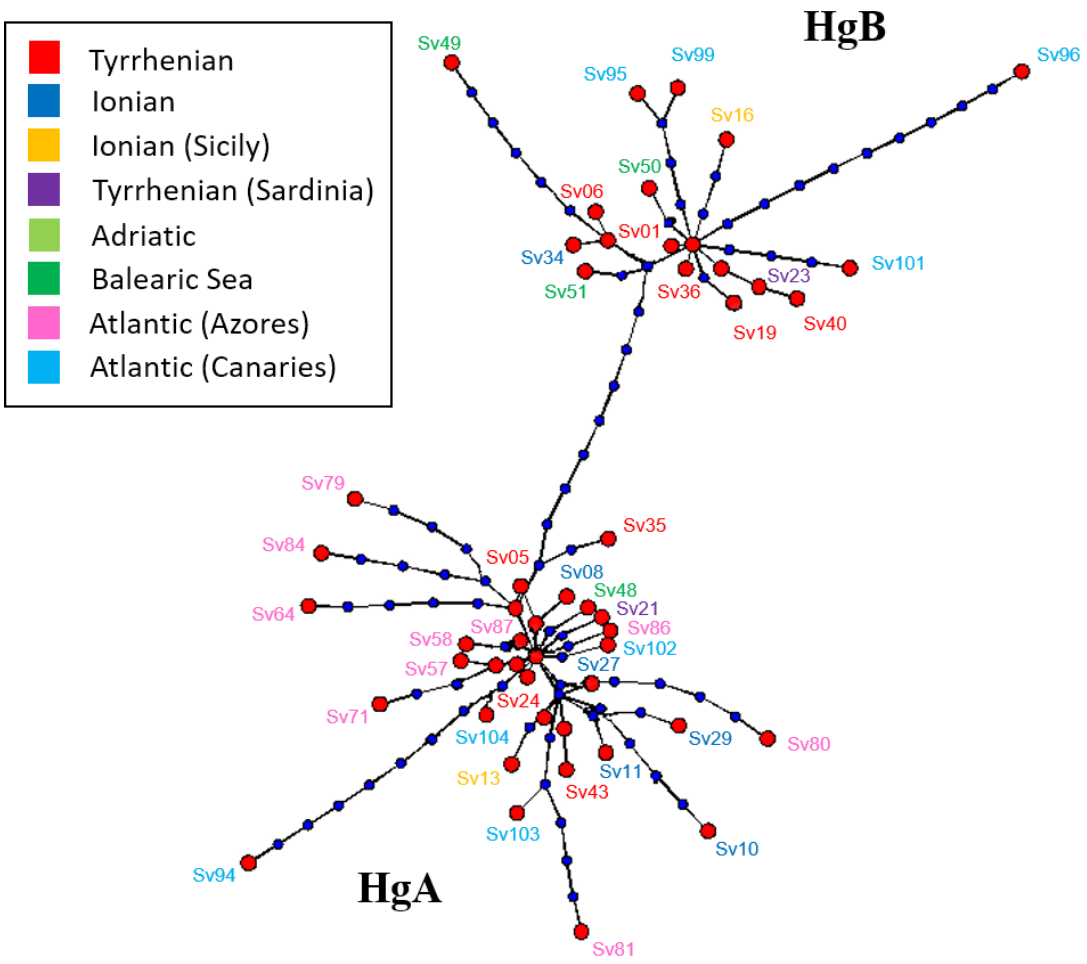

Figure 3.11: Haplotype network for *S. viridensis* mtDNA CR haplotypes. Individual labels are coloured by geographic origin. Blue dots represent nucleotide substitutions between haplotypes. Two clear haplogroups are present and labelled HgA and HgB.

3.3.2 Stable Isotope analyses ($\delta^{15}N$ and $\delta^{13}C$) of Sphyraena viridensis tissues

Analysis of stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) was conducted on the tissues of 103 individuals. Where individuals had both muscle tissue and a fin clip available the fin clip was given analysis preference, though for Azorean samples both tissue types were analysed to make a tissue comparison – see below. Excluding dual tissue samples for the Azores, the primary data set consisted of 44 muscle samples and 59 fin clips. It is well documented that stable isotope ratios can vary between tissue types (Ben-David and Flaherty, 2012) and examination of values obtained from muscle and fin clips revealed that to be true in this study (Figure 3.12). Data values did not meet parametric assumptions (data not normal, Anderson-Darling test, $p<0.005$, $p<0.005$ and $p=0.019$ for $\delta^{13}C$ muscle, $\delta^{15}N$ fin and $\delta^{15}N$ muscle respectively) so were tested non-parametrically. Significant differences were detected between muscle and fin clip samples for both $\delta^{13}C$ and $\delta^{15}N$ (Kruskal-Wallis, $p=0.005$ and $p=0.002$ respectively).

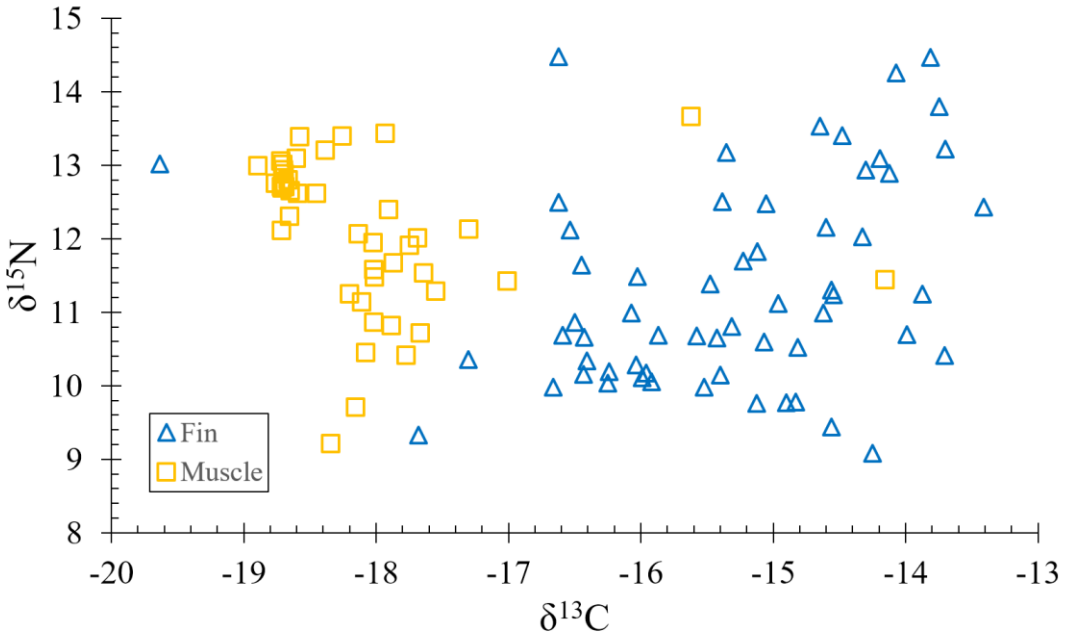

Figure 3.12: Plot of $\delta^{15}N$ vs $\delta^{13}C$ for all samples showing the clear dichotomy between tissue types, muscle being depleted for ^{13}C.

This difference in isotope ratios between tissue types is considerably larger than that observed in *Tursiops truncatus* (Chapter 2, Section 2.3.3) but crucially the number of samples from a single location (Azores) with a mix of tissue types is greater in *S. viridensis* (n=34) so an attempt at applying a discrimination factor was made (Figure 3.13). To do this I adjusted muscle $\delta^{13}C$ and $\delta^{15}N$ values by the equation describing the line connecting the mean fin and mean muscle values (y=-0.77x-1.501). This discrimination factor, although approximate, allowed a sensible geographical comparison of stable isotope values when this would have been otherwise impossible (Figure 3.14).

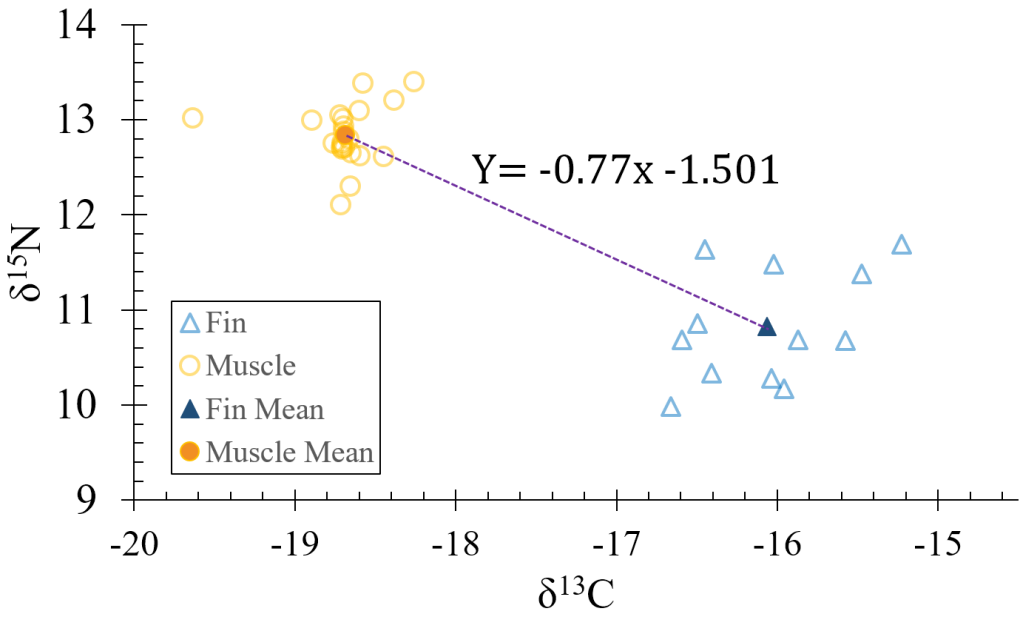

Figure 3.13: Plot of $\delta^{15}N$ vs $\delta^{13}C$ for fin (△) and muscle (○) from Azorean samples. Cluster means are shown in bold colour. The line represents the discrimination factor applied to muscle tissue for further analysis.

Examination of stable isotope values in *S. viridensis*, after application of the aforementioned discrimination factor, revealed some fairly strong geographic partitions (Figure 3.14, Figure 3.15). Most notably, the Atlantic sample sites (the Azores and the Canary Islands) suggest evidence of lower trophic level feeding, with lower values of $\delta^{15}N$, than a number of Mediterranean locations. In particular, samples from the Ionian Sea returned significantly higher $\delta^{15}N$ values (Kruskal-Wallis, p=0.005). Although *S. viridensis* from both Atlantic sample sites exhibited isotopic signatures suggestive of lower trophic level feeding than most Mediterranean sites they were significantly different from each other in values for $\delta^{15}N$ (Kruskal-Wallis, p=0.000 in both cases) meaning they likely do not share a common diet. The lower $\delta^{13}C$ values observed in Azorean *S. viridensis* than those found in much of the Mediterranean likely represents the contrasting environment (pelagic vs coastal) from which samples derived. *S. viridensis* from the Azores also demonstrated a much lower level of variation in $\delta^{15}N$ and $\delta^{13}C$ than those found in the Mediterranean, potentially suggesting a more specific diet and less generalist approach to feeding and more homogenous environment respectively (see Figure 3.15, Coefficient of Variation (CV) for $\delta^{13}C$ and $\delta^{15}N$ was -2.10, 3.90 and -6.38, 10.34 for Azores and Ionian locations respectively). Samples from Sicily had high values for $\delta^{15}N$ but showed large variation in $\delta^{13}C$ value. It is possible that *S. viridensis* from both Marseille and the nearby Balearic island of Mallorca are feeding on a common prey source and inhabit a similar environment as no significant difference was detected in their stable isotope signatures for either $\delta^{13}C$ or $\delta^{15}N$ (ANOVA, p=0.250 and p=0.775 respectively).

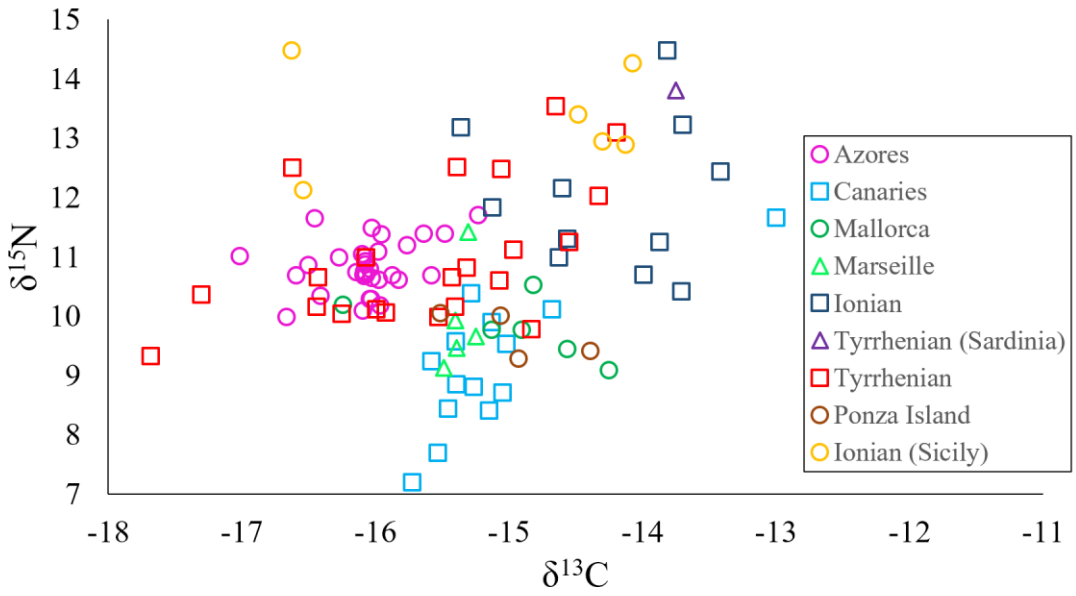

Figure 3.14: Plot of $\delta^{15}N$ vs $\delta^{13}C$ for all samples following application of the tissue discrimination factor to convert muscle to equivalent fin values.

Isoscapes for *S. viridensis* were generated using data from all samples but with the discrimination factor applied. For both the $\delta^{13}C$ and $\delta^{15}N$ isoscapes the lack of sample around the Iberian Peninsula results in a break in the isoscape projection in this region. As with *Tursiops truncatus* (Figure 2.24) there is a general pattern of higher values in coastal waters and lower values in pelagic habitats in the $\delta^{13}C$ isoscape (Figure 3.16). Particularly high values (-12.5‰), indicating enrichment of ^{13}C, were observed around the coast of Sicily, likely influenced by a single individual from Isola delle femmine on the island's northern coast that had a $\delta^{13}C$ of -11.5‰. As predicted by Figure 3.14, the $\delta^{13}C$ isoscape predicts a shared isotopic signature for *S. viridensis* from Marseille and the Balearic Islands.

The isoscape for $\delta^{15}N$ (Figure 3.17) depicts the previously detected significant difference in values between Azorean *S. viridensis* and those found in the Canaries. There is a large area of high $\delta^{15}N$ value in the Ionian Sea and particularly in the Gulf

of Taranto with $\delta^{15}N$ values reaching over 14‰. A similar region of enrichment for ^{15}N is seen off the west coast of Sardinia but with values only reaching around 13.2‰.

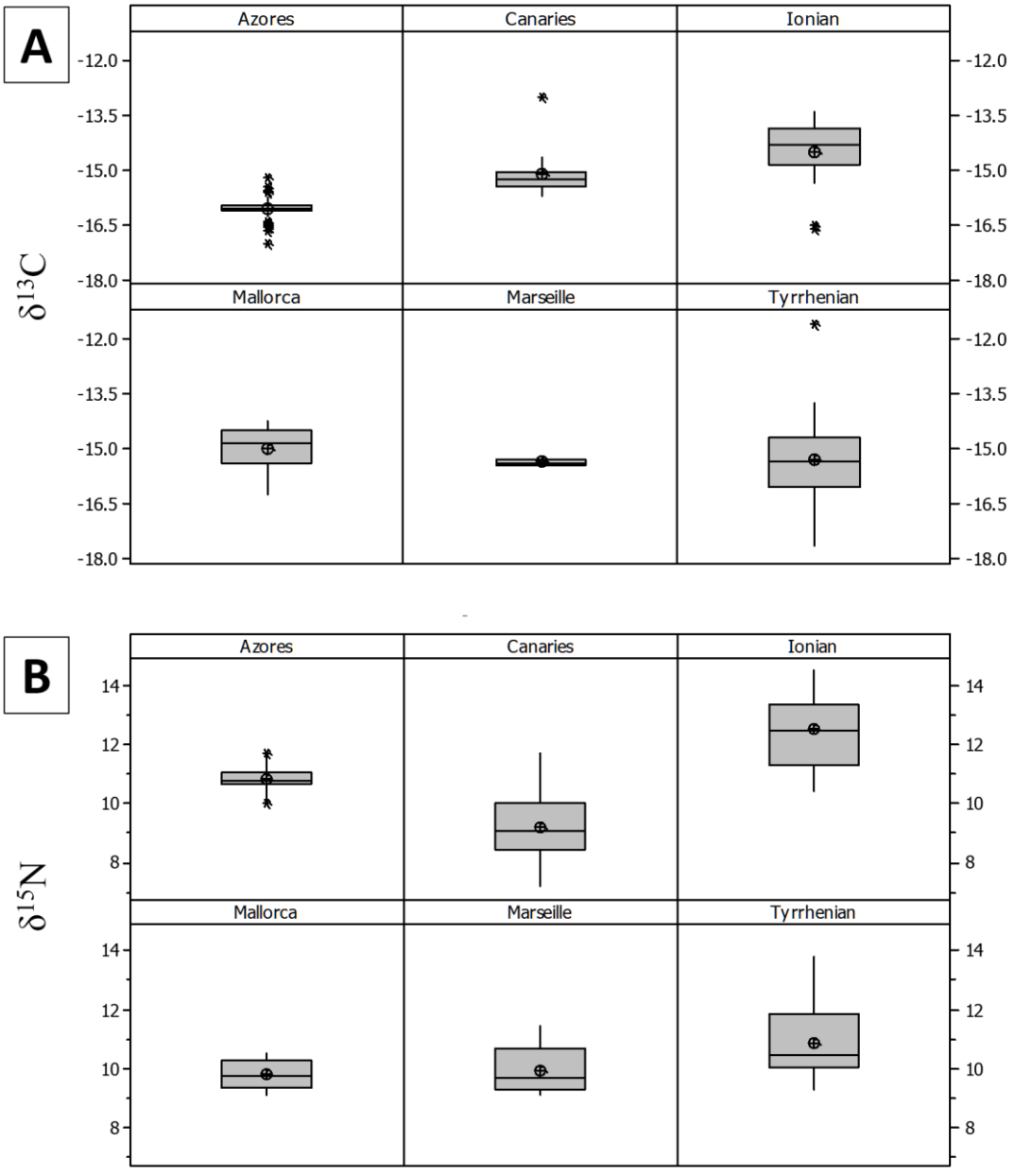

Figure 3.15: Boxplots of geographically collated $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values from *S. viridensis* tissue samples

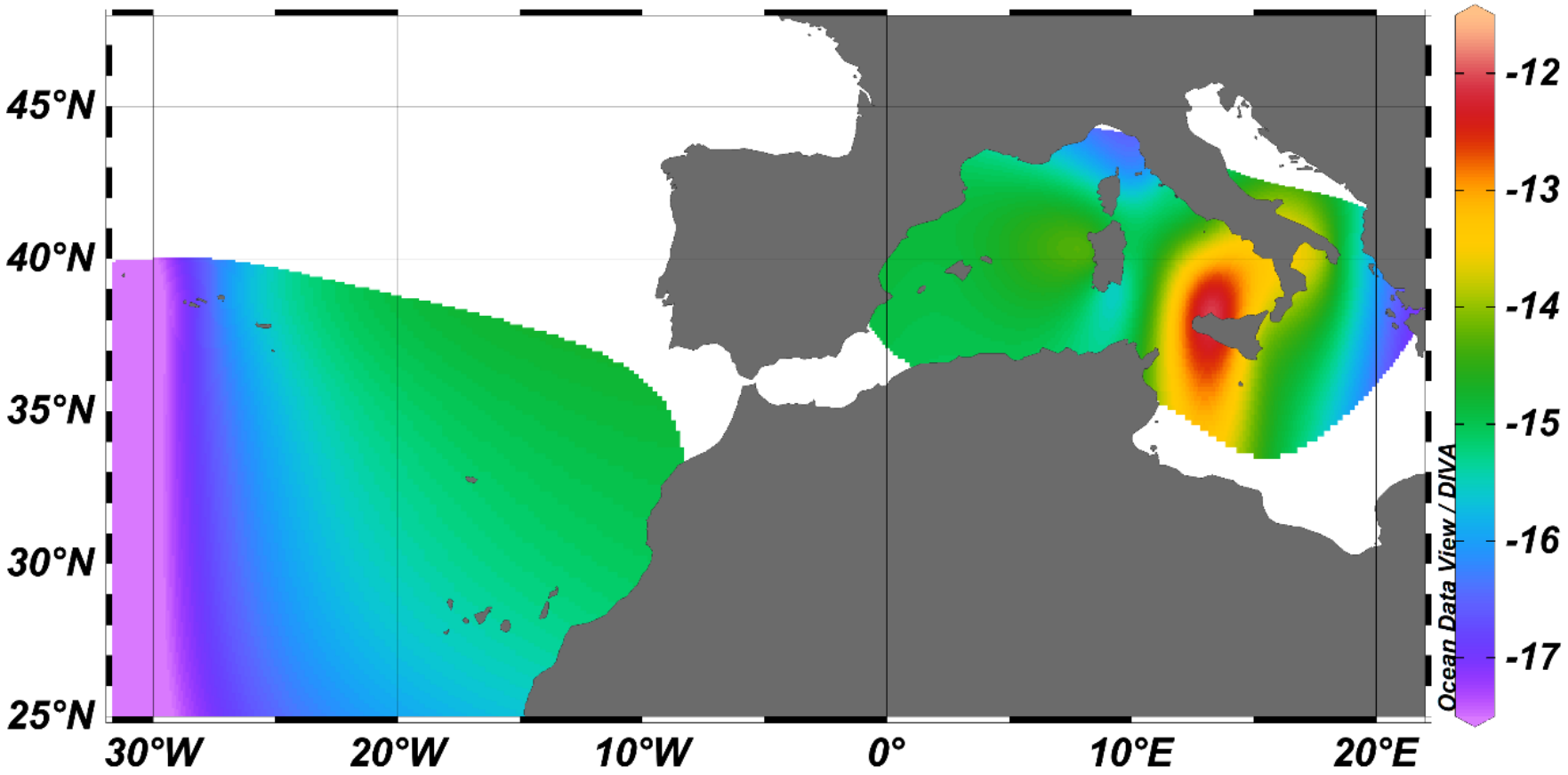

Figure 3.16: Isoscape for $\delta^{13}C$ generated from tissue samples of *Sphyraena viridensis*. Isoscape plotted using *Ocean Data View*.

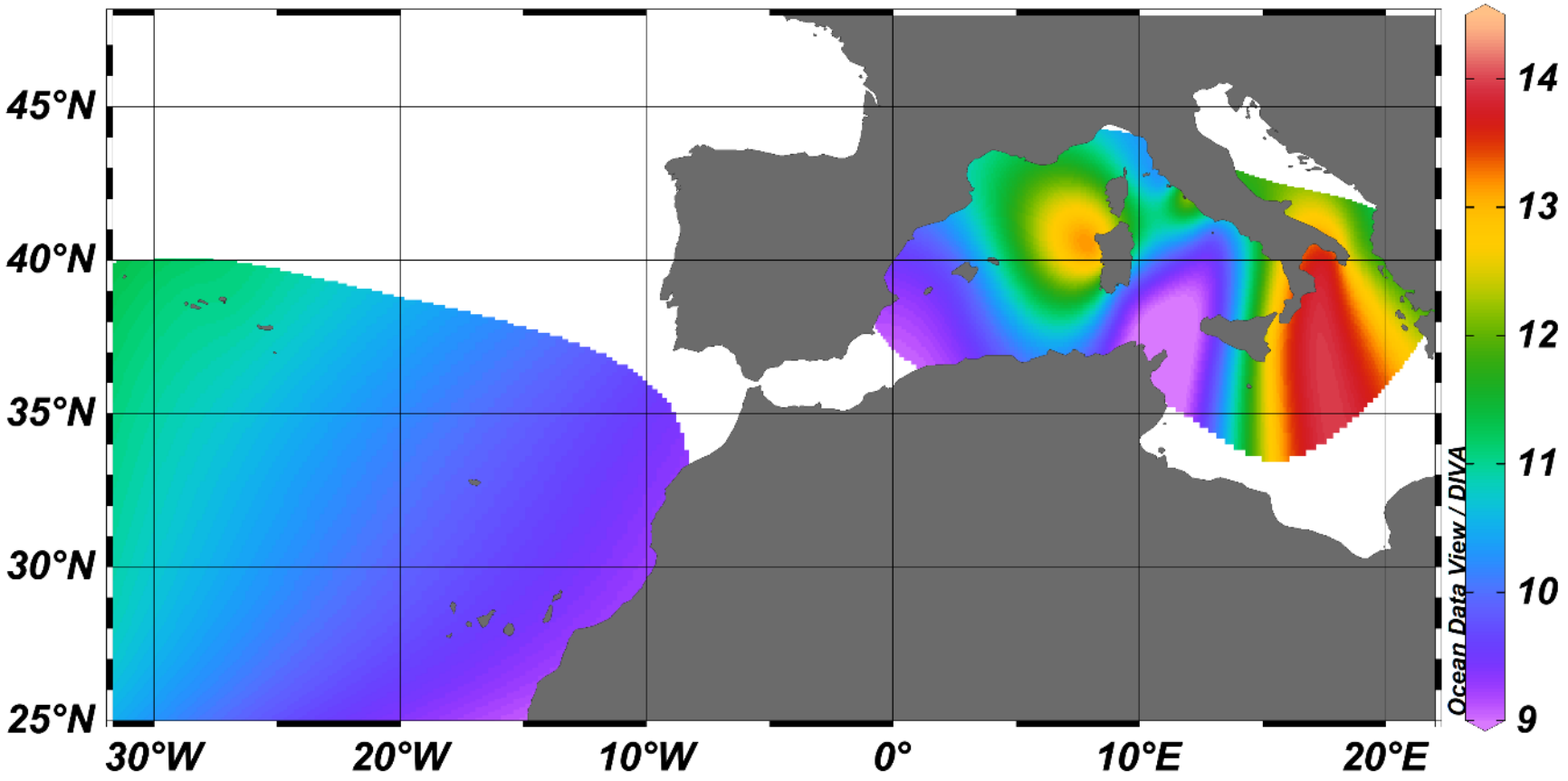

Figure 3.17: Isoscape for $\delta^{15}N$ generated from tissue samples of *Sphyraena viridensis*. Isoscape plotted using *Ocean Data View*.

3.3.3 Integration of genetic and environmental data

Mantel tests (Table 3.5) were conducted to examine correlation between genetic distance (Jost's pairwise D) and potential influencing factors (SST, Salinity, Chl A, $\delta^{13}C$ and $\delta^{15}N$). For these tests all individuals are treated as a single stock rather than discrete populations and the examination is for correlation between overall genetic variation and potential factors and are thus not subject to the same low reliability encountered with investigations of population differentiation via F_{ST} and Φst. However, all tests returned non-significant results. There was thus no evidence of Isolation By Distance (IBD) or correlation with any environmental variables or diet (as ascertained from stable isotope values). It is perhaps interesting to note that correlation between genetic variation and both salinity and $\delta^{15}N$ was almost significant (p=0.058 and p=0.066 respectively). Data relationships for these two tests are displayed in Appendix VI. Data were tested for differences in stable isotope values between haplogroups and no significant difference was found for either $\delta^{13}C$ (Mann-Whitney U test, P = 0.6841) or $\delta^{15}N$ (Mann-Whitney U test, P = 0.3929).

Table 3.5: Results of Mantel tests of correlation between matrices of Pairwise D between populations and environmental variable matrices. No parameters returned a significant correlation with genetic distance.

Env. variable	Mantel Statistic	P-value	Significant?
Geographic distance	0.5656	0.083	N
SST	0.0104	0.433	N
Salinity	0.5066	0.058	N
Chlorophyll A	-0.1804	0.558	N
$\delta^{13}C$	-0.2411	0.858	N
$\delta^{15}N$	0.3569	0.066	N

Genetic correlation with potential explanatory factors (SST, Salinity, $\delta^{13}C$ and $\delta^{15}N$) was further investigated with redundancy analysis (RDA) (Figure 3.18). RDA revealed that environmental and dietary factors explained 4.6% of the overall genetic variance, of which RDA1 was the most significant contributor – making up 44% of this value and this is significant at $p<0.004$. RDA2 contributed 27% of the overall explainable variance and was just significant at $p=0.05$. Within RDA1 SST and Salinity are the largest contributing factors. Collinearity between factors was checked through investigation of variance inflation in *R* package *Vegan* and although SST and Salinity showed high values (7.6 and 8.3 respectively) this is within the generally recognised acceptable upper limit of 10 (Zuur *et al.*, 2010).

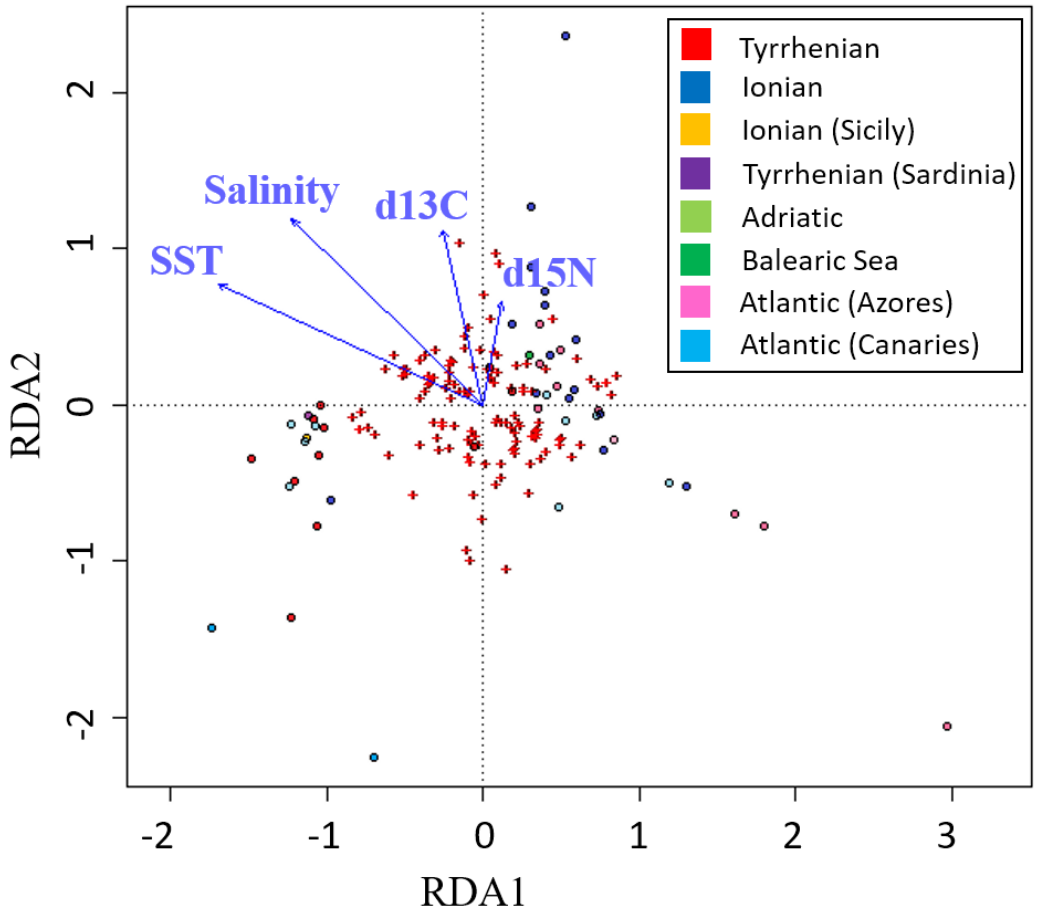

Figure 3.18: Redundancy Analysis (RDA) plot investigating genetic variance explained by environmental and dietary factors. RDA1 axis explains 2.1% of all genetic variance ($p<0.001$), with salinity and SST being major contributing factors to this axis.

3.4 Discussion

Analysis of mtDNA in this chapter demonstrated that, despite low sample sizes in some regions, the Yellowmouth Barracuda *Sphyraena viridensis* has detectable geographic population structure across the area covered in this study. There were no geographical regions that displayed a distinct genotype but limited lineage sorting is sometimes reported in mtDNA analyses, even being documented in *Tursiops truncatus* (Natoli *et al*., 2004). However, there was clear evidence for two clear haplogroups, as seen in Milana *et al.* (2014). Additional samples collected for this study all displayed haplotypes that readily fit into these two haplogroups so it is likely that only these two haplogroups are present in the geographic coverage of this study. Whether further haplogroups exist in other parts of the range of *S. viridensis* (i.e. northern Atlantic and far eastern Mediterranean) is unknown.

Milana *et al.* (2014) estimated that divergence between these two haplogroups likely occured during either the Riss or Würm glaciations (approximately 300-130kya and 115-11.7kya respectively), based on a mutation rate of either 3.6% per million years (a generally accepted teleost mutation rate (Donaldson and Wilson, 1999)) or 13% per million years (the highest observed in teleosts (Brown *et al*., 1993)). This study, with its increased geographical coverage, demonstrated that there was unequal distribution of haplogroups across the species range. For example, there were no individuals from haplogroup B in the Azores and the majority of Tyrrhenian individuals came from haplogroup B. Though cautiously interpreted, this potential Atlantic-Mediterranean split could be a result of historical changes in oceanography with current distribution being a result of secondary contact as suggested by Milana *et al.* (2014). A possible mechanism for this divergence could be a restriction in larval and adult passage through the Gibraltar Strait. During the Würm glaciation the

Gibraltar Strait was considerably more restricted than the present day with the strait being shallower and extending a further 25 miles in to the Atlantic (Anderson, 1965) which could have increased its capacity as a barrier to gene flow for a number of species. Indeed, the Gibraltar Strait has been implicated as a barrier to gene flow in a number of teleost species (Bargelloni *et al*., 2005; Blanquer *et al*., 1992; Borsa *et al*., 1997; Suzuki *et al*., 2004). However, the complete lack of available *S. viridensis* samples from the Iberian Peninsula means that the true point of divergence cannot currently be identified and the possibility that the Almería-Oran front, rather than the Gibraltar Strait, is the actual barrier remains plausible as suggested for other teleosts (Cimmaruta *et al*., 2005; Galarza *et al*., 2009; C. Schunter *et al*., 2011a).

An alternative hypothesis, albeit a potentially less likely mechanism, is that the dichotomy of haplotypes represents a staged colonisation of the Mediterranean from an Atlantic origin following the Messinian Salinity crisis. Santini *et al.* (2015) suggests that *S. viridensis* diverged from all other barracuda species around 5.33 million years ago which is incidentally the same time estimate given to the Zanclean Flood which refilled the much-desiccated Mediterranean (Abril and Periáñez, 2016). The re-filling of the Mediterranean occurred in stages, with each basin filling sequentially (Periáñez and Abril, 2015), with some authors suggesting that this could have taken sufficient time for divergence to occur (Meijer and Krijgsman, 2005). Under this hypothesis the geographical mixing of haplotypes observed today would be a result of contemporary currents dispersing larvae and the limited larval retention potential of ocean fronts (Naciri *et al*., 1999). However, numerous elements limit the likelihood of this mechanism. Firstly, recent modelling investigations have suggested that the refilling of the Mediterranean occurred in months rather than thousands of years (Garcia-Castellanos *et al*., 2009), certainly too little time for genetic divergence to occur.

Secondly, within the significant time since the Zanclean flood it is very likely that genetic signals of sequential colonisation would be lost, given the higher mutation rate observed in mitochondrial DNA than in nuclear DNA (Chenoweth *et al*., 1998) combined with potential for many localised population divergences and the potential for significant dispersal since this period. It should also be considered that physical events at not always necessary for mtDNA divergence as this can occur stochastically. Finally, this model cannot readily explain the presence of both haplogroups in the Atlantic population of the Canaries.

The Tyrrhenian showed considerable evidence of limited mixing with other regions, both through dominance of haplogroup B and in measures of genetic distance between it and other regions. Although some caution should be taken given the implications of low sample size on F_{ST} and Φ_{ST} (Holsinger and Weir, 2009), the general trend is likely to be real and is supported by pooled sample comparisons (Table 3.4). The Tyrrhenian has several circulating currents (Artale *et al*., 1994) that has been shown to have an isolating effect of species that have a planktonic larval stage (De Innocentiis *et al*., 2004), such as *S. viridensis*. Surface currents are known to impact larval distribution (Cuttitta *et al*., 2016) and thus ultimately influence adult population structure (Carlsson *et al*., 2004) and I would propose a very similar influence of such currents on larval dispersal for *S. viridensis*.

Surface water currents may also be able to explain the mix of haplogroups observed in *S. viridensis* individuals from the Canary Islands. Running north to south along the Atlantic coast of North Africa is the Canary Current (Mason *et al*., 2011), an eastern boundary current that makes up the eastern section of the North Atlantic Gyre. This current could readily transport individuals and more importantly larvae from both the Azores (along with the Azores current) and any escapees from the

Mediterranean that pass westwards through the Gibraltar Strait down to the Canaries. The Canary Current is recognised as a major transport system of fish larvae for a number of species (Brochier *et al*., 2008). Similarly, the Algerian current, running eastwards along the north coast of Africa, could be responsible for transporting *S. viridensis* larvae from the Atlantic to the Ionian Sea, thus providing a mechanism for the low levels of differentiation between the Azores and the Ionian suggested by F_{ST} and Φ_{ST} values.

This study revealed the first evidence of environmental factors being a driver for genetic variation within *S. viridensis* with RDA suggesting that they could explain up to 4.7% of the observed variance. Although small, this value is well within the range of environmentally explained genetic variance seen in other landscape genetics studies (Harrisson *et al*., 2017; Riordan *et al*., 2016). Within the principal explanatory axis (RDA1) SST and salinity were major contributing factors (salinity was also the primary contributor to the just significant RDA2 as well as nearly a significant factor in the lower powered Mantel tests). Although variation in salinity is known to greatly influence the growth and survival of larval fish (McCarthy *et al*., 2020) I would posit that this result is a representation of the salinity differential between the Atlantic and, in particular the eastern, Mediterranean and the unequal distribution of haplogroups, rather than an impact on physiology.

Within the family Sphyraenidae there is a precedent for high levels of larval dispersal with high gene flow between ocean basins. The Great Barracuda *Sphyraena barracuda* has been shown to demonstrate this pattern of high dispersal and connectivity, with its lack of genetic population structure likened to that of many large oceanic predators rather than structure seen in reef-dependent teleost species (Daly-Engel *et al*., 2012). *S. barracuda* is often associated with nearshore environments

(O'Toole *et al*., 2010) and in all cases where samples were obtained for this study *S. viridensis* individuals were similarly associated with coastal reefs or islets. Though typically sleeker, the mean length of *S. viridensis* used in this study (91.4cm) was comparable to the typical adult length of *S. barracuda* so, though based on more than size alone, it could be presumed that their capacity for movement in mature individuals is similarly comparable.

Villegas-Hernández *et al*. (2014) suggests that *S. viridensis* is a thermophilic species that is found predominantly in the north-west Mediterranean but this study clearly demonstrates that this species is able to thrive in cooler Atlantic waters (Azores). The actual range of *S. viridensis* is not known to be greater than the waters highlighted in Figure 3.1, and this may be conservative due to sampling bias, but given the large variation in environmental conditions across this range and the possibility of aforementioned high dispersal potential for this species it is intriguing as to why, like *S. barracuda*, it has not become a more cosmopolitan species.

Use of mtDNA, as well as allozymes, has revealed considerable interspecific variation in the effect of dispersal capability on population structure. For example, there have been studies that have revealed much greater population structure than their dispersal capability would suggest, even over large distances (Ovenden *et al*., 2004; Palumbi, 1994). Alternatively, there have been those studies that reveal very little evidence of population structure irrespective of distances involved (Lacson, 1992; Shulman and Bermingham, 1995). Within teleost species genetic variation tends to be higher in habitat specialists but lower in those species that are more generalist (Smith and Fujio, 1982). Our understanding of the ecology of *S. viridensis* is undoubtedly still in its infancy and it may yet be too early to say with much authority whether this species can be classed as a habitat generalist or specialist. However, the relatively low

level of genetic divergence between the geographically distant Azores and Ionian Sea and its adaptation to the range of diets seen in this study may push it closer to a generalist classification.

The discrepancy observed between tissue types for stable isotope values in this study is commonplace in ecological studies (Hobson, 1993; Kurle *et al*., 2014; Logan and Lutcavage, 2010; Piola *et al*., 2006) and to be expected given our understanding of differential tissue turnover rates in teleosts (Madigan *et al*., 2012). However, the relationship between stable isotope values obtained from different tissue types is known to be species specific and value conversions via linear equations cannot be readily transferred from one species to another (Willis *et al*., 2013). As such, conducting such experiments as presented herein, with tissue conversion equations presented, is vital for the future study of *S. viridensis*.

The low levels of variation in stable isotope values in Atlantic individuals in this study could suggest that *S. viridensis* found in these cooler waters are making a compromise on their thermophilic preferences (Villegas-Hernández *et al*., 2014) in return for a specific prey source. Equally, the targeting of specific prey, rather than the generalist approach suggested for most Mediterranean *S. viridensis*, would suggest an environmental adaptation at the edge of the species range, albeit one which is not yet fully reflected in their genotypes. A similar specific dietary signal was observed for those samples derived from the coastal waters of Marseille where all samples originated from an artificial reef (Cresson *et al*., 2014). Though Cresson *et al.* (2014) suggests that isotope signatures from species feeding on complex artificial reefs reflects that found in natural habitats the authors did not have access to the geographical range of data for *S. viridensis* as presented in this study, thus areas of greater dietary variability as reported here should not be surprising.

Significantly higher values of both $\delta^{15}N$ and $\delta^{13}C$ were found for samples from the Ionian Sea than from the Azores. Whilst the low levels of variation within the Azorean samples indicate exploitation of a specific prey species and the low values derive from a prey species that is at a relatively low trophic level, we must also consider what is causing the increased values of $\delta^{15}N$ and $\delta^{13}C$ for Ionian Sea individuals, relative to other areas. The higher $\delta^{13}C$ found in Ionian Sea individuals is possibly driven by a more coastal association, with individuals spending increased time closer to the shoreline, as well as being driven by higher temperatures (Michener and Kaufman, 2007). However, it could be expected that a more coastal lifestyle would result in a depletion of ^{15}N rather than an enrichment due to the typical terrestrial water runoff being ^{15}N depleted. That this is overcome, and significantly so, must indicate a higher trophic level predation for Ionian Sea *S. viridensis* and I thus accept my second hypothesis. This must be ground-truthed with stomach contents analysis and future studies should make this a priority.

Though significant differences in $\delta^{15}N$, indicative of differential trophic feeding, were detected this was not reflected in the observed genetic data, $\delta^{15}N$ being only a very minor contributor to significant RDA axes and thus bringing into question the hypothesis that diet specialisation influences population structure. However, the major environmental factors that contributed most to significant RDA axes (SST and Salinity) typically define the geographical localities of each population and its basin/sea. Furthermore, such factors could play a measurable role in the distribution of prey resources. We must also consider that the genetic dataset analysed, being derived from matrilineal mtDNA, though it may reflect genetic divergence after a significant period of time of limited geneflow between populations, is unlikely to represent local adaptation to prey resources as nuclear markers may.

Studies on other complete predatory teleost species in the Mediterranean have suggested higher trophic level feeding, as suggested by $\delta^{15}N$, than that found in *S. viridensis* (Rogdakis *et al*., 2010; Stergiou and Karpouzi, 2002). Although some studies have recorded *S. viridensis* feeding on cephalopods and crustaceans (Barreiros *et al*., 2002; Kalogirou *et al*., 2012), they are predominantly piscivorous. It is thought that the lower values for $\delta^{15}N$ reported in *S. viridensis*, as seen in this study, compared to other piscivorous teleost species, is created by a preference for this species to predate on smaller planktivorous species (Cresson *et al*., 2014). However, the $\delta^{15}N$ values seen in *S. viridensis* are comparable to those seen in *Tursiops truncatus*, in particular the putative Azores-Sicily metapopulation, suggesting that they feed on similar prey and thus increasing confidence in its suitability as a comparative species for investigations into the environmental drivers of population structure in marine predators.

Such plasticity to different diets and environments could hint of as yet undetected species-wide genetic diversity as adaptability is often pre-indicated by increased genetic diversity, though this does vary by species, environment and population (Booy *et al*., 2000). A successful future endeavour to compile a SNP data set for this species would be useful in determining this diversity and help understand how it is able to adapt to these varied environments. Furthermore, such a dataset would also provide a higher resolution examination of population structure potentially revealing any subtle structure not revealed by mitochondrial DNA (Morin *et al*., 2004) and help overcome some of the issues created by lower sample sizes (Willing *et al*., 2012).

The unsuccessful outcome of the ambitious attempt to develop a bait-capture microsatellite library means that I am unable to robustly test hypothesis 1, as laid out

at the beginning of this chapter. However, based on mtDNA alone the evidence for unequal distribution of haplogroups across the Atlantic and Mediterranean taken with significant and substantial measures of divergence (F_{ST} and Φ_{ST}) I reject a suggestion of panmixia and instead accept my first hypothesis. It is possible that even greater levels of gene flow may be detectable using bi-parental markers (i.e. SNPS or microsatellites) in future studies.

The evidence for population structure in *S. viridensis*, as outlined above, was in part significantly explained by environmental factors. This forces me to, albeit cautiously, accept my third hypothesis that *S. viridensis* genetic structure is influenced by environmental variables or gradients. Currents clearly play a major role in both aiding and limiting of gene flow but ocean fronts may also play a role as the Atlantic-Mediterranean divergence in haplotype distribution could implicate the Almería-Oran front. Clearly further investigation is needed and interpretation would be greatly aided by a higher resolution dataset, both in sample distribution and genetic marker choice.

3.6 Conclusion

This chapter presented evidence for population structure within *Sphyraena viridensis*. Whilst some suggestion can be made of an Atlantic-Mediterranean divergence with a potential limited barrier to gene flow along the coast of the Iberian Peninsula, sample sizes for populations and their distribution prevent hard conclusions from being drawn.

However, the geographic distribution of mtDNA haplotypes showed some correlation with environmental factors, principally salinity, and this likely reflects the ability of ocean basins to retain both adults and larvae via surface currents. Surface currents and gyres likely play a fundamental role in both the mixing (the Canary Islands) and the isolation (Tyrrhenian Sea) of *S. viridensis* populations.

The isotopic evidence presented herein suggests the *S. viridensis* is highly adaptable and in some geographic regions, particularly at the edge of its range, is likely a prey specialist.

Future work should approach from two angles, the first being to increase sample coverage in key areas, such as around the Iberian Peninsula and the South coast of Italy as well as expanded coverage to include the far eastern Mediterranean and north Atlantic, and secondly to develop a higher-resolution dataset using next generation sequencing technologies. The method development in Appendix V lays the foundation for one such approach using microsatellite markers but a SNP library would also greatly aid in furthering our knowledge of this species.

Environmental influences on the evolution of the genus *Tursiops* and the role of ecotypes in speciation

4.1 Introduction

There is now overwhelming evidence for the presence of two distinct ecotypes of *Tursiops truncatus*: a wide ranging and genetically diverse offshore ecotype and a low ranging coastal ecotype with relatively low genetic diversity (Barros *et al*., 2010; Costa *et al*., 2016; Fruet *et al*., 2017; Hoelzel *et al*., 1998; Lowther-Thieleking *et al*., 2015; Perrin *et al*., 2011; Rossbach and Herzing, 1999; Segura *et al*., 2006; Torres *et al*., 2003). These ecotypes of *T. truncatus* have been observed throughout the western hemisphere and although not seen in every occurrence of *T. truncatus*, where they are observed there are consistently ecological, genetic and morphological differences between them (though these differences do vary geographically).

However, in the eastern hemisphere these ecotypes seem absent and instead it has been suggested that the Indo-Pacific Bottlenose Dolphin, *Tursiops aduncus* (Ehrenberg 1833), fills the niche of the coastal ecotype (Hawkins and Gartside, 2008; Tezanos-Pinto *et al*., 2009). This habitat specialisation, between ecotypes in the western hemisphere and species in the eastern hemisphere, suggests that although social structure and communication are likely important to the formation of population structure in bottlenose dolphins, as suggested in Chapter 2, the environment still plays a fundamental role in the evolution of the genus *Tursiops*. Phylogenetic analyses

suggest that the more coastal *T. aduncus*, or at least a coastal common ancestor, is the predecessor to *T. truncatus* and that climate oscillations, by direct influence or proxy, led to the derivative offshore and wide ranging *T. truncatus* that then expanded its range to populate the world (Moura *et al*., 2020, 2013). Studies of the *T. truncatus* mtDNA genome suggest that the coastal ecotype of *T. truncatus*, all Mediterranean *T. truncatus* and the sub species Black Sea *T. truncatus ponticus* were later derived from the offshore *T. truncatus* (Gaspari *et al*, 2015; Moura *et al*., 2013). Indeed, my findings in Chapter 2 support the notion that coastal *T. truncatus* in the Mediterranean are derived from the offshore ecotype as gene flow is strong from the putative offshore Sicily-Atlantic metapopulation out to inshore regions (Figure 2.19b).

There are many remaining questions regarding the evolutionary history of the genus *Tursiops*. *Tursiops* and the wider Delphinidae are moderately well represented in the fossil record (Barnes, 1990) but there are significant inequalities in the geographic distribution of fossil evidence and the timing of a number of speciation/radiation events remains uncertain. The current ecotypes observed in *T. truncatus* provide an extant proxy for the possible mechanism of speciation between *T. truncatus* and *T. aduncus*, that is to say differential habitat specialisation (offshore and coastal). As such, examination of current differences and levels of admixture between these ecotypes may provide valuable insight into cetacean evolution as well as improve our understanding of these populations in ways that could be useful for their conservation and management.

This chapter will investigate the level of genetic differentiation and gene flow between *T. truncatus* offshore and coastal ecotypes and will then seek to examine the historical speciation between the *T. aduncus* and *T. truncatus* species and the possible environmental influences involved.

4.1.1 Differences between the offshore and coastal ecotypes of Tursiops truncatus

In Chapter 2 I briefly introduced the key differences between the accepted ecotypes of *Tursiops truncatus*, offshore and coastal (sometimes alternatively referred to as pelagic and nearshore respectively) (Lowther-Thieleking *et al*., 2015; Perrin *et al*., 2011; Rossbach and Herzing, 1999) and as a principal focus of this chapter I shall now consider this in greater detail. The offshore ecotype is principally found in pelagic waters, often many hundreds of miles from land; however, they are sometimes found close to shore, even displaying a distribution overlap with coastal ecotype groups (Simões-Lopes *et al*., 2019). This is often the case where there are deep waters close to shore, such as around offshore volcanic islands or where the continental shelf edge or underwater canyons lie close to the coast (Klatsky *et al*., 2007; Milmann *et al*., 2017).

It has been suggested that the offshore ecotype is better adapted for deeper diving, as may be required to hunt for prey in a deep-water environment and comparisons of the morphology and physiology of the two ecotypes supports this hypothesis (Hersh and Duffield, 1990). It has been found that the offshore ecotype has higher levels of haemoglobin (Duffield *et al*., 1983) as well as much higher levels of haematocrit (red blood cell density) (Fahlman *et al*., 2018b; Klatsky *et al*., 2007; Schwacke *et al*., 2009), thus improving their capability for the deeper dives they have been observed to undertake (Mate *et al*., 1995). When hybrid offshore-coastal *T. truncatus* have been reared in captivity they have been found to have intermediate haematocrit and haemoglobin values, suggesting that at least some of this adaptation is genetically derived (Duffield *et al*., 1983).

Morphologically the two ecotypes can often be quite readily differentiated. It should be noted, however, that this is not universally true and in in some locations

other features, described below, are used to define ecotype. Generally the offshore ecotype is observed to have darker colouration, often with a very falcate dorsal fin; whereas the coastal ecotype is generally lighter coloured and with a less pronounced falcate dorsal fin (Félix *et al*., 2018; Simões-Lopes *et al*., 2019). However, these features have not been shown to be defining of ecotype in the same way for north east Atlantic individuals (Evans pers. comm. 2020). A number of differences in skeletal anatomy have been observed between the two ecotypes (Costa *et al*., 2016; Hoelzel *et al*., 1998; Mead and Potter, 1995; Perrin *et al*., 2011; Toledo, 2013), some of which are thought to support deeper diving in offshore *T. truncatus* (Klatsky *et al*., 2007). However, there is geographic discrepancy in which ecotype obtains the larger body size. In the Gulf of California the coastal ecotype is generally larger and more robust (Segura *et al*., 2006) but in the north western Atlantic the offshore ecotype is the larger of the two (Klatsky *et al*., 2007). Larger body size may support deeper diving as a larger muscle mass would increase storage capacity of O_2 (Fahlman *et al*., 2018a).

The ecotypes also differ socially. For example, it has been noted that the offshore ecotype has an increased tendency to form large groups of individuals (n>100), whereas the coastal ecotype is more likely to be observed in smaller pods of twenty individuals or less (Salinas-Zacarias, 2005). Off the coast of Ireland it has been observed that the inshore ecotype has a cohesive fission-fusion social organisation where individuals typically occupy large home ranges; whilst offshore individuals tend to form multiple and smaller distinct social groups, possibly coming together to form the aforementioned larger groups (Oudejans *et al*., 2015). Whether these observations hold true for other ecotype populations in the region is as yet not fully understood.

A number of studies have now explored the idea that the separation of these two ecotypes is not just a geographical one but also an example of dietary niche specialisation. Exploitation of different food resources between the ecotypes has been demonstrated by Stable Isotope Analysis (SIA) (Barros *et al*., 2010; Dıaz-Gamboa, 2003; Segura *et al*., 2006), stomach content analysis (Hoelzel *et al*., 1998; Mead and Potter, 1995) and even studies of their teeth which showed morphological divergence that is consistent with the differential prey targets suggested by SIA (Perrin *et al*., 2011).

There is no apparent single universal genotype for either the *T. truncatus* offshore or coastal ecotype. However, regional differentiation between ecotypes has been demonstrated in a number of localities (Fruet *et al*., 2017; Hoelzel *et al*., 1998; Lowther-Thieleking *et al*., 2015; Moura *et al*., 2020; Segura *et al*., 2006). Furthermore, coastal populations are also often locally differentiated, even between adjacent coastal regions (e.g. Fruet *et al*., 2014; Sellas *et al*., 2005). The level of differentiation, in both phenotype and genotype, has led some to suggest that the two ecotypes could form valid parapatric sub-species (*T. truncatus gephyreus* [coastal ecotype] and *T. truncatus truncatus* [offshore ecotype]) (Costa *et al*., 2016) or even valid species (*T. gephyreus* and *T. truncatus*) (Wickert *et al*., 2016). Recent genomic studies have suggested that the designation of coastal *T. truncatus* as a subspecies of the offshore ecotype may be most appropriate (Moura *et al*., 2020). This study will build on the findings of Moura *et al*. (2020) in examining admixture between the two ecotypes to further our current understanding.

The ecotypes of *T. truncatus* found off the east coast of North America have been relatively well studied (Hoelzel *et al*., 1998; Mead and Potter, 1995, 1990; Pate and McFee, 2012; Torres *et al*., 2005, 2003). Offshore *T. truncatus* along this coast

reach as far north as the state of Maine, whilst the coastal ecotype, though present, is less common this far north. The species is occasionally reported in Canadian Atlantic waters, though with increasing rarity (Baird *et al*., 1993). Along the US coast the coastal ecotype shows a distinct seasonality to their distribution – occupying waters as far north as New Jersey in the summer months but no further north than Cape Hatteras in the winter (Mead and Potter, 1990). The offshore ecotype in this region appear to occupy waters which progress further northwards than the coastal ecotype, likely supported by the warmer waters of the gulf stream that flows along this coast at a distance concurrent with the distribution of offshore individuals. There is a region of sea, perhaps 80-100 km wide, between the two distributions where sightings of *T. truncatus* are relatively rare (see Figure 4.2). The delineation of ecotypes appears to fall geographically within this gap; Torres *et al*. (2003) suggested that all dolphins found within 7.5 km of the shore were of the coastal ecotype and that all found greater than 34 km from the shore were of the offshore ecotype. This fits with earlier studies that suggested that the 25m isobath may be the limit of the coastal ecotype (Kenney, 1990). The offshore ecotype in this region appears to show several adaptations that could support deeper diving, including greater nareal diameter and higher haematocrit and red blood cell count (Hersh and Duffield, 1990). These adaptations would support feeding in offshore *T. truncatus* in this region, who predominantly feed on deep water squids and myctophids (Mead and Potter, 1990).

The ecotypes within this region have been defined not just on appearance and morphometrics but genetically as well. Hoelzel *et al*. (1998) examined genetic differentiation using mtDNA and microsatellite markers in 29 coastal and 26 offshore *T. truncatus* taken from the east coast of North America. This study found not only genetic differentiation between ecotypes in both markers but also demonstrated greater

genetic diversity in the offshore population. This genetic differentiation was further reinforced by an extensive study of mtDNA from 304 individuals which demonstrated that the ecotypes could be easily defined by genetic data (Torres *et al*., 2003). Furthermore, the coastal ecotype has since been shown to have local subdivision into groups that show genetic differentiation (Richards *et al*., 2013).

Modern genomic techniques now provide an opportunity to re-examine this genetic division between *T. truncatus* ecotypes and crucially to better understand levels of ongoing geneflow between the two.

4.1.2 T. truncatus vs T. aduncus; macro-differences in morphology

Only two full species of Bottlenose Dolphin are widely accepted in the scientific community: the Common Bottlenose Dolphin *Tursiops truncatus* and the Indo-Pacific Bottlenose Dolphin *Tursiops aduncus.* The Burrunan Dolphin *T. australis* is proposed as a valid third species (Charlton-Robb *et al*., 2011) but is not yet formally accepted (Committee on Taxonomy, 2018). *T. australis* is relatively geographically limited and shares many of the same characteristics as *T. aduncus*, indeed recent genetic evidence suggests it should only be considered as a subspecies to *T. aduncus* (Moura *et al*., 2020). The Black Sea population of *T. truncatus* is largely accepted only as a subspecies of *T. truncatus - Tursiops truncatus ponticus* (Viaud-Martinez *et al*., 2008).

T. truncatus is known to grow significantly larger than *T. aduncus,* reaching a length of around 4m where it occurs in colder waters whereas *T. aduncus* is only thought to reach a maximum of 2.6m when fully grown (Shirihai *et al*., 2006). However, it should be noted that *T. aduncus* is limited to tropical/sub-tropical waters

and *T. truncatus* present in similar latitudes show no significant increase in size. The rostrum of *T. aduncus* is proportionately longer to its body size than that of *T. truncatus* but overall skull length is generally longer in *T. truncatus* (Hale *et al*., 2000). These morphological parameters are known to exhibit sexual dimorphism in *T. truncatus* but no sexual dimorphism has been observed in *T. aduncus* (Hale *et al*., 2000; Read *et al*., 1993).

In most instances *T. aduncus* has more teeth (23-29 on each side of the jaw) than *T. truncatus* (18-27 on each side of the jaw) (Jefferson *et al*., 2015) and this has been linked to an apparent difference in prey species as shown by stomach contents analysis of the two morphotypes found off the coast of South Africa – now believed to be *T. truncatus* and *T. aduncus* living parapatrically (Ross, 1977). Visibly, *T. aduncus* and *T. truncatus* can often be distinguished by colouration and skin spotting (Figure 4.1). Spotting on the ventral surface is particularly common in *T. aduncus* throughout its range but by contrast is relatively rare in *T. truncatus* with the possible exception of older females (Gridley *et al*., 2018).

There have also been recorded differences in their acoustic characteristics. Although their source parameters remain similar, it has been observed that *T. aduncus* has both increased frequency and greater directionality in its clicks than *T. truncatus*, even when environmental context is taken in to account. It is thought that this may be possible due to species differences in the morphology of the air sacs and soft structures of the melon (Wahlberg *et al*., 2011).

T. aduncus is limited to warmer temperate and tropical regions of the Indo-Pacific and only in coastal habitat, yet *T. truncatus* has an almost cosmopolitan distribution including cool temperate and pelagic waters. It is thought this adaptation

to offshore waters was crucial in the divergence of these two species and the evolution of the genus (Moura *et al*., 2020). Increasing our understanding of the divergence of these two species, in the context of contemporary environment and climate events, is crucial to improving our understanding of the key drivers of evolution in cetaceans.

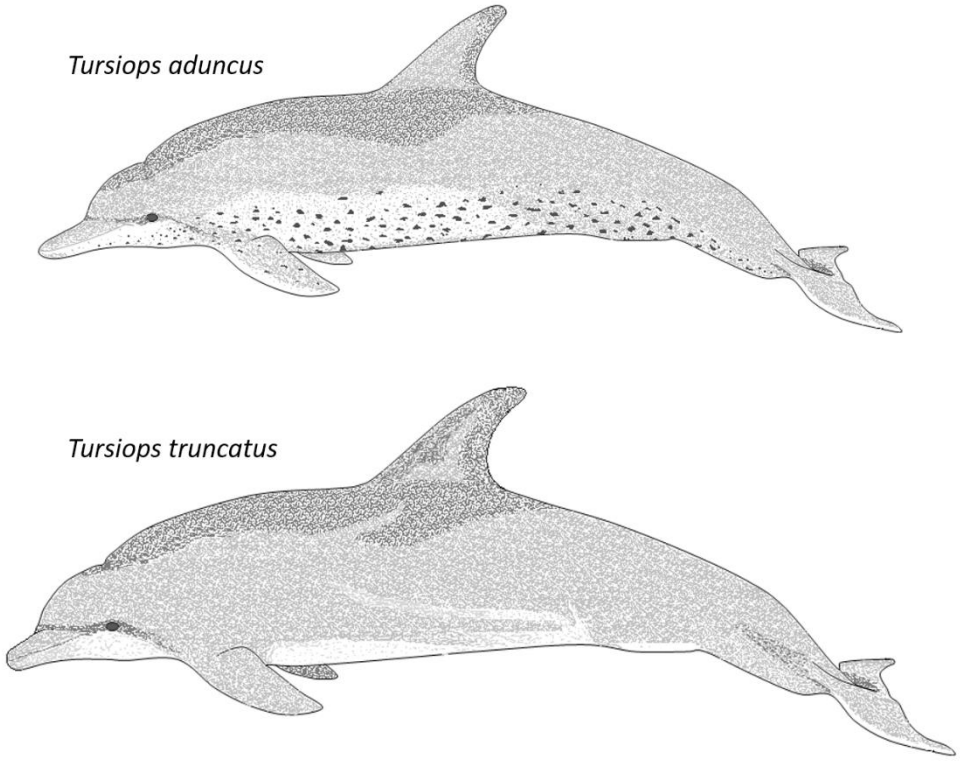

Figure 4.1: External morphology of *Tursiops aduncus* (above) and *Tursiops truncatus* (below). Note the longer rostrum and ventral spotting in *Tursiops aduncus* and the more falcate dorsal fin and distinctive lighter stripe on the dorsal flank that is common in many *Tursiops truncatus*. © Daniel Moore

4.1.3 Evolution of the genus Tursiops

The genus *Tursiops* emerged from the Delphininae during the late-Miocene, perhaps around seven million years ago (Barnes, 1990). Whilst it had been proposed that the genus had a Mediterranean origin owing to the abundance of fossil examples

found in this region (Barnes, 1990), in particular the Italian ranges, this has now been dismissed as molecular evidence indicates that all extant species likely had an Australasian (or at least wider Oceanian) origin (Moura *et al*., 2013).

Since the separation of *Tursiops* from the Delphininae in the late Miocene there have been at least six species accepted by the scientific community, two of which are extant and detailed earlier in this chapter. The first fossil evidence of an extinct *Tursiops* identified to species level came in the form of skeletal remains found at Colle della Torrazza, Italy, in 1793 by Giuseppe Cortesi. These remains were named *Delphinus cortesii* before being reassigned to the genus *Tursiops* in 1891. This holotype of *Tursiops cortesii* was discovered in rocks of the Piacenzian stage of the Pliocene, making it between 2.58 and 3.6 million years old, and at least 1.7 million years after the Zanclean flood that opened up the Mediterranean for inhabitation by cetaceans. There have since been at least ten further specimens of this species found, all from Italy, though most are either lost or destroyed (Barnes, 1990). Although this is the first appearance of *Tursiops* in the fossil record it is highly probable that the genus was in fact present in other geographic areas, though possibly in pelagic regions hence the lack of fossils discovered thus far as deep-water sediment exposures are far less common. Nevertheless, this may represent the first foothold that the genus established in the Mediterranean.

The second fossil species of *Tursiops* to be recognised today is *Tursiops astensis* (Sacco, 1891). Though originally assumed to be a variety (or subspecies) of *T. cortesii*, it is now established as a species in its own right owing to the significant differences in cranial structure, in particular that of the cranial crests. *T. astensis* has been found in rock beds dating to the early Late Pliocene (3-3.5 million years ago) in Italy.

A third described species is also derived from rock deposits in Italy, *Tursiops capellini* (del Prato, 1898), dating to about 3.5 million years ago. It is believed that the two discovered examples of this species have now been lost by Italian museums but they were well illustrated, allowing its description as a species separate from *T. cortesii* and *T. astensis*, largely owing to the increased number of teeth found in either jaw of *T. capellini.* The large number of *Tursiops* species found in the Mediterranean during this time period illustrate high levels of taxonomic diversity, possibly due to the rapidly changing environment during this early period of colonisation.

The final recognised and formally named fossil species of *Tursiops* is *Tursiops osennae* (Simonelli, 1911) and although the holotype was also found in Italy (Sicily) it is from much later rock deposits of around 0.5-0.8 million years old (early Late Pleistocene). Though physically larger than modern *T. truncatus*, the fossil examples of *T. osennae* have 21 teeth either side of the rostrum which places it well within the modern range observed in *T. truncatus* (18-27 (Jefferson *et al*., 2015)). This has led some authors to suggest that *T. ossenae* may be ancestral to *T. truncatus* and *T. aduncus* (Pilleri, 1985). However, morphological features of other skeletal components, principally the radius and ulna which are longer than those of *T. truncatus*, indicate that *T. osennae* is too derivative to be a direct ancestor of *T. truncatus* (Barnes, 1990). It would therefore seem that *T. osennae* was merely an ecological predecessor to *T. truncatus* in the Mediterranean before a later invasion of the latter that then out-competed and replaced the former.

There have been other fossils attributed to the genus *Tursiops* though not yet identified to species level. These include examples of *Tursiops* sp. from the Yorktown formation of the Lee Creek mine in Virginia, USA dating to the early Pliocene (4.8-3.0 million years ago) (Whitmore, 1994) and from the Capistrano Formation of Orange

County, California USA, (2-4 million years ago) (Barnes, 1976). These examples show that the genus had a cosmopolitan distribution even before the deposition of the numerous fossil species seen in the Mediterranean area.

Fossils attributed to *T. truncatus* have been found across the current species range, including the North Sea (van der Kortenbout, 1983; van Netten and Reumer, 2009), US eastern states (Blake, 1939) and the Asian Pacific coast (Tsao, 1978). All fossils attributed to *T. truncatus* come from beds dating across the Pleistocene but without any geographic progression in emergence, indicating that the species likely achieved its wide-ranging distribution relatively rapidly after emergence. Unfortunately, the fossil record is still insufficient in being able to date and track that spread or support the molecular evidence for an Australasian (Moura *et al*., 2013) or more likely wider Indo-Pacific Ocean origin (Moura *et al*., 2020).

It is thought that the common ancestor of the extant *Tursiops* lineages occupied a coastal habitat somewhere in Oceania and likely spread around the Indian and Pacific Ocean basins via the nearshore habitats. At a time currently unknown, it is believed that *T. truncatus* made its first appearance as a pelagic derivative of this coastal ancestor and then colonised first the offshore Atlantic environment before adapting to coastal Atlantic regions as well as the Mediterranean (Moura *et al*., 2013). Molecular evidence suggests that the presence of *T. truncatus* in the Indo-Pacific is likely the result of a secondary invasion as samples of this species around Australasia group closely with those found in the north west Atlantic offshore population (Hoelzel *et al*., 1998; Möller and Beheregaray, 2001; Natoli *et al*., 2004). It is now well acknowledged that the specialism between different habitats, coastal and offshore, which are often embodied through the presence of ecotypes is the principal driver of divergence in *Tursiops* spp. (Moura *et al*., 2013). This is why a thorough examination of the genetic

relationship between extant ecotypes is so crucial to our understanding of this process. It is proposed that cyclical climate patterns, principally glaciation, are likely a key driver in the formation of these habitat specialisms (and ultimately species) (Hoelzel, 1998). Various mechanisms have been proposed for this, from the physical opening and closing of coastal habitat due to sea level changes (Moura *et al*., 2013) to spatio-temporal habitat differentiation due to monsoon patterns (Gray *et al*., 2018).

Although some attempts at understanding the timing of divergence between the extant *Tursiops* lineages has been attempted before (Moura *et al*., 2013), which estimated divergence in the late Pleistocene, clearly a more refined and accurate dating of this event could better place it within an environmental context and thus provide better inferral of causality. I earlier proposed that the extant lineages diverged around one million years ago (Moura *et al*., 2020) and here I expand on those analyses to better place these results in an environmental context as well as further apply Next Generation Sequencing technologies to estimate divergence and historic gene flow.

4.1.4 Aims and hypotheses

This chapter examines the level of genetic differentiation between the offshore and coastal ecotypes of *T. truncatus* found off the eastern seaboard of the United States of America. It is thought that habitat specialisation may have been a pathway in the speciation of *T. truncatus* and *T. aduncus* and understanding this process between ecotypes of *T. truncatus* will further our understanding of evolutionary drivers across the genus. Following this I will examine the *T. aduncus*/*T. truncatus* speciation more closely, seeking to pinpoint the timing of this event, placing it in an environmental and

palaeontological context. Finally, I will investigate past and present gene flow between the two species.

Towards these aims this chapter will examine and test the following hypotheses:

Hypothesis 1:

H1: There is clear genetic differentiation between offshore and coastal ecotypes of *Tursiops truncatus* found off the eastern seaboard of the United States of America.

Hypothesis 2:

H1: There is observable levels of gene flow between offshore and coastal ecotypes of *Tursiops truncatus* found off the eastern seaboard of the United States of America.

Hypothesis 3:

H1: There is evidence that environment played a significant role in the *T. aduncus*/*T. truncatus* speciation event.

4.2 Methodology

4.2.1 Genome data for T. aduncus and T. truncatus

Production of a sequenced genome from *Tursiops aduncus* was completed by Prof. Rus Hoelzel for analyses conducted in Moura *et al*. (2020). DNA was extracted from a sample of a *T. aduncus* individual originating from South Africa. A total of 2µg of purified DNA was used to create the library, which was shotgun sequenced on a single lane of an Illumina HiSeq 2500, with version 4 chemistry. The library was created using the Illumina PCR-free Tru-Seq kit following manufacturer's instructions.

T. truncatus genome data were downloaded from NCBI (SRA accession number SRX200685). These data came from a female *Tursiops truncatus*, believed to be a US military captive animal, that was originally collected from the north west Atlantic Ocean. The sample was Sanger sequenced at ~2X coverage by the Human Genome Sequencing Center at the Baylor College of Medicine and the Broad Institute, under the guise of the BCM-HGSC Marine Mammal Genome Projects. Later refinements and revisions were made via shotgun sequencing.

4.2.2 ddRADseq of offshore vs coastal T. truncatus

In order to investigate the genetic structure, divergence and admixture of the offshore vs coastal ecotypes of *Tursiops truncatus* this study made use of samples taken from the east coast of North America, originally used in a previous investigation (Hoelzel *et al*., 1998). 22 samples were classified as coming from the offshore ecotype and came from a mix of strandings and offshore bycatch. All offshore specimens that came from bycatch were caught close to the continental shelf margin, approximately

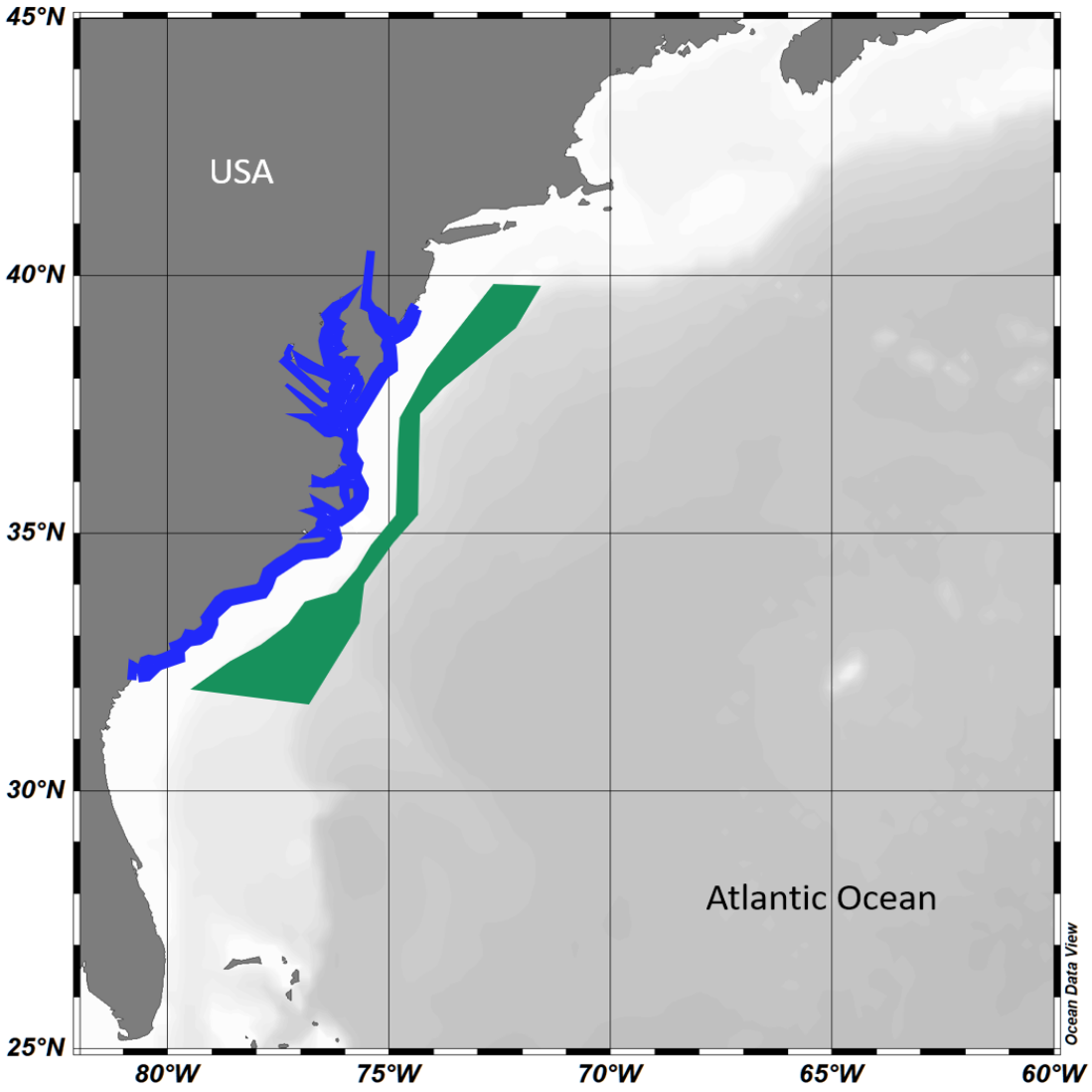

Figure 4.2: Distribution of samples of *T. truncatus* off the USA eastern seaboard. Coastal samples were retrieved from either strandings or within five miles of the coast (Blue). Offshore samples were caught as by-catch around 100-300 miles offshore, along the continental shelf edge (Green).

100-300 miles offshore from the states of Maine, Massachusetts, New Jersey, North Carolina and Georgia. 21 samples came from dolphins classed as coastal ecotype, all of which were obtained from individuals that were either stranded or live caught for display purposes from waters close to shore. There is a slim possibility due to archive management issues that some samples came from further south, in the waters off Florida, but still occupying a coastal-offshore distribution. As such, they remain suitable for inclusion in this study. Stranded coastal ecotype specimens came from the

states of Georgia, New Jersey, North Carolina, Virginia and Maryland. Stranded animals were identified to ecotype based on species composition of stomach contents and parasites (Hoelzel *et al*., 1998; Mead and Potter, 1995), with most animals being classified by one or both of these criteria. The DNA for all Western North Atlantic (WNA) population samples was pre-extracted and held in an archive at the Department of Biosciences, Durham University. Preparation of genetic libraries for sequencing followed Peterson *et al.* (2012). For a comprehensive methodology please see Chapter 2, section 2.2.5, and for a desktop protocol see Appendix I.

4.2.4 Bioinformatic analysis

The following methodologies contain core information and chapter specific details only. For more information and explanations of specific analyses please see section 2.2.9.

Illumina sequencing data was deposited on the Hamilton Cluster at Durham University. Raw sequencing reads were demultiplexed and quality filtered using the *process radtags* subprogram in *Stacks* v1.35 (Catchen *et al*., 2013) *Process radtags* flags were defined as *-q 10 -t 92 -r –renz_1 msp1 –renz_2 hindIII -E phred33*. All reads were trimmed to a length of 92 nucleotides.

Reference indices were created using the *bowtie2-build* command within *Bowtie2* v2.2.5 (Langmead and Salzberg, 2012) using the *Tursiops truncatus* reference genome Tur_tru_Illumina_hap_v1 (GenBank Accession GCA_003314715.1). Sequence alignment was completed using Bowtie2 v2.2.5 with default settings. The *Stacks* v1.35 (Catchen *et al*., 2013) *ref_map.pl* pipeline with flags set as *-m 3 -n 2* was

used for SNP detection. Only samples with greater than 900,000 reads and less than 30% missing data were included in analysis.

Population structure analyses were conducted using Principal Component Analysis (PCA) (Jolliffe, 2011), Discriminant Analysis of Principal Components (DAPC) (Jombart *et al*., 2010), *Admixture* (Alexander *et al*., 2009) and a method of ancestral probability based on Minor Allele Frequency (MAF) using the *R* package *SambaR* (de Jong *et al*. unpubl.). Analysis of contemporary migration rates (gene flow) was calculated using *BayesAss3-SNPs* (Mussmann *et al*., 2019) and visualised in *R* using *SambaR*. Analyses were conducted on all loci that passed quality filters.

Analyses of genetic diversity (genome wide heterozygosity, Minor Allele Frequencies (MAF) calculations etc.) were conducted using *SambaR* (de Jong *et al*. unpubl.). Analysis of genetic differentiation between populations (Nei's genetic D, Weir and Cockerham's F_{ST} etc.) was conducted using the *R* package *hierfstat* (Goudet, 2005).

Estimations of the D-statistic (ABBA-BABA statistic), to assess evidence of ancestral introgression between the offshore and coastal ecotypes – as well as between these ecotypes and other groups within the genus *Tursiops*, were conducted using *Dsuite* (Malinsky *et al*., 2019). *Dsuite* estimates D-statistics for all population trios in the dataset against a fixed outgroup – in this case a SNP dataset of the Rough-Toothed Dolphin *Steno bredanensis* from Moura *et al.* (2020). Additional SNP datasets were included including a number from Chapter 2 of this thesis (Azores, Cádiz, Black Sea, and Mediterranean (West Italy)). Further datasets derive from Moura *et al.* (2020) (South Africa, China and Burrunan).

To estimate species divergence time within the genus *Tursiops*, specifically between *T. truncatus* and *T. aduncus,* I calculated ancestral population size (N_e) over time using the Pairwise Sequential Markovian Coalescent (PSMC) model, utilising the software package *PSMC* (Li and Durbin, 2011). Time of divergence was approximated from the point of convergence of the ancestral effective population sizes between the two species, when each were plotted on the same axes. For input to this analysis I used genome sequence data of *T. truncatus* downloaded from NCBI (SRA accession number SRX200685). This data, along with the *T. aduncus* genome data previously outlined, were then mapped to the Ensembl reference genome for *T. truncatus* (turTru1.92) using *Bowtie* v2.2 (Langmead and Salzberg, 2012). All read duplicates were removed with *SAMtools* (Li *et al*., 2009). Final N_e plots were produced using the Perl scripts for *PSMC*, with 64 atomic time slots and 28 free interval parameters. -P was set to '4+25*2+4+6'. Mutation rate and generation time were both derived from available literature with mutation rate set at $1.5x10^{-8}$ (Moura *et al*., 2014) and generation time at 21.5 years (Taylor *et al*., 2007). 100 bootstraps were performed with 5Mb sequence segments randomly selected and resampled by replacement.

PSMC relies on the expectation that N_e for the now separate populations will deviate at the point of divergence. However, it is theoretically possible that N_e for each population may remain similar, at least for a period of time, and thus lead to erroneous estimates of divergence time. To qualify our estimate of time of divergence *PSMC* was also run with pseudo-diploid genomes (h*PSMC*) (Li and Durbin, 2011; Prado-Martinez *et al*., 2013). To create pseudo-diploid genomes, two haploid sequences from each species, with loci of low consensus quality (<20) excluded, were hybridised using the program *SEQTK* (Li, 2012). Mitochondrial sequences were excluded throughout all *PSMC* analyses.

Ancestral population sizes, divergence times and migration rates were calculated from the *T. truncatus* and *T. aduncus* genomes using the Generalised Phylogenetic Coalescent Sampler (*G-PhoCS* (Gronau *et al*., 2012)), which implements a Bayesian coalescent approach. An outgroup was utilised to increase robustness of the *T. truncatus* and *T. aduncus* divergence estimate, consisting of the Pacific White-sided Dolphin *Lagenorhynchus obliquidens* with the genome downloaded from GenBank (Accession number GCA_003676395.1). *L. obliquidens* was selected due to the phylogenetic distance between it and the genus Tursiops (Moura *et al*., 2020). *G-PhoCS* calculates posterior probabilities for migration rates and divergence times based on genomic data under the assumption that loci located along the genome, that are inputted in the form of multiple sequence alignments, are separate and neutrally-evolving. A Markov Chain Monte Carlo (MCMC) sampling method is utilised to sample genealogies and model parameters for each input locus. *G-PhoCS* parameters were set at default settings with migration bands placed between all species and in all directions for investigative purposes. Sampling was conducted on a data subset (one in every 10,000 bases) to expedite analysis. The MCMC was run with 25,000 burn-in iterations and 100,000 additional iterations.

4.3 Results

4.3.1 Genetic differentiation between ecotypes of T. truncatus in the western North Atlantic

Examination of genetic diversity between offshore and coastal *T. truncatus* of the Western North Atlantic (WNA) found the greater proportion of segregating sites in the offshore ecotype as well as the greater proportion of heterozygote sites (Figure 4.3A-B). Comparisons of Allele Frequency Spectra between coastal and offshore ecotypes (Figure 4.3C) revealed that the offshore ecotype had the highest proportion of polymorphic sites for smaller Minor Allele Frequency (MAF) classes but the lowest proportion in larger classes, the reverse being true for the coastal ecotype. Although they displayed similar levels of nucleotide diversity, examinations with Watterson's theta showed that whilst the coastal ecotype displayed a depletion of rare alleles, the offshore ecotype had an excess (Figure 4.3D). Minor allele frequencies were similar for both ecotypes (Figure 4.3E) but the coastal ecotype did present higher frequencies for the lower minor allele classes.

Principal Component Analysis (PCA) revealed a genetic distinction between the two ecotypes with 93% of individuals projected exclusively within the same ecotype ellipses as their assigned ecotype (Figure 4.4). This differentiation was also demonstrated by Discriminant Analysis of Principal Components (DAPC), analysed with retention of the first 40 principal components, with two clearly separated peaks in Euclidean space (Figure 4.5). Explorations of assignment probability (Figure 4.6) also supported a clear genetic distinction between the ecotypes. Interestingly, two individuals (C5 [Coastal] and P4 [Offshore]) assigned entirely to the opposite ecotype to which it was presumed they had come from. At least seven further individuals showed partial assignment to their opposing ecotype, suggesting some degree of

admixture. This was truer for presumed-coastal ecotype individuals than for those that were presumed to be offshore ecotypes.

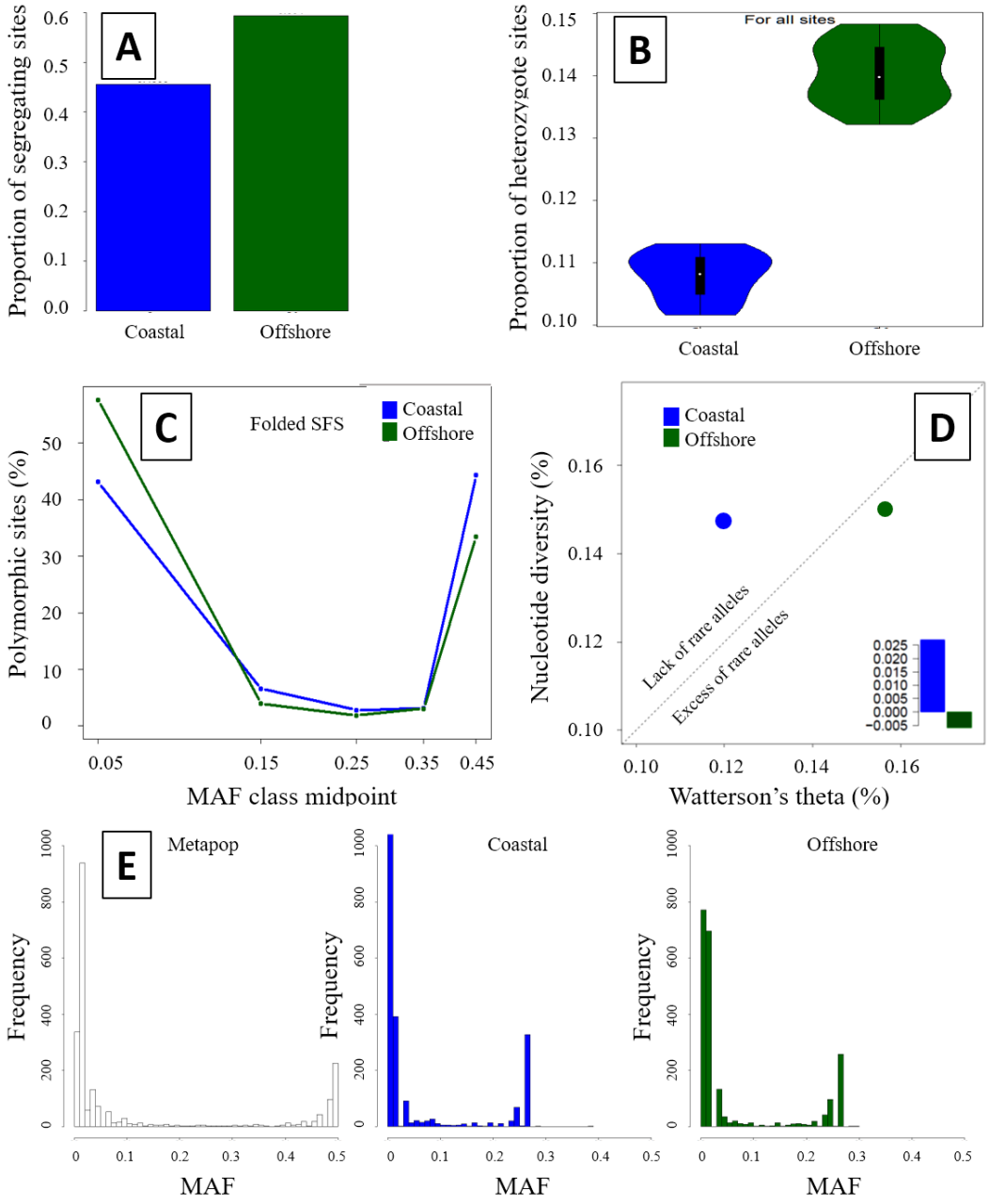

Figure 4.3: Genetic diversity of the WNA Offshore and Coastal populations of *Tursiops truncatus*.

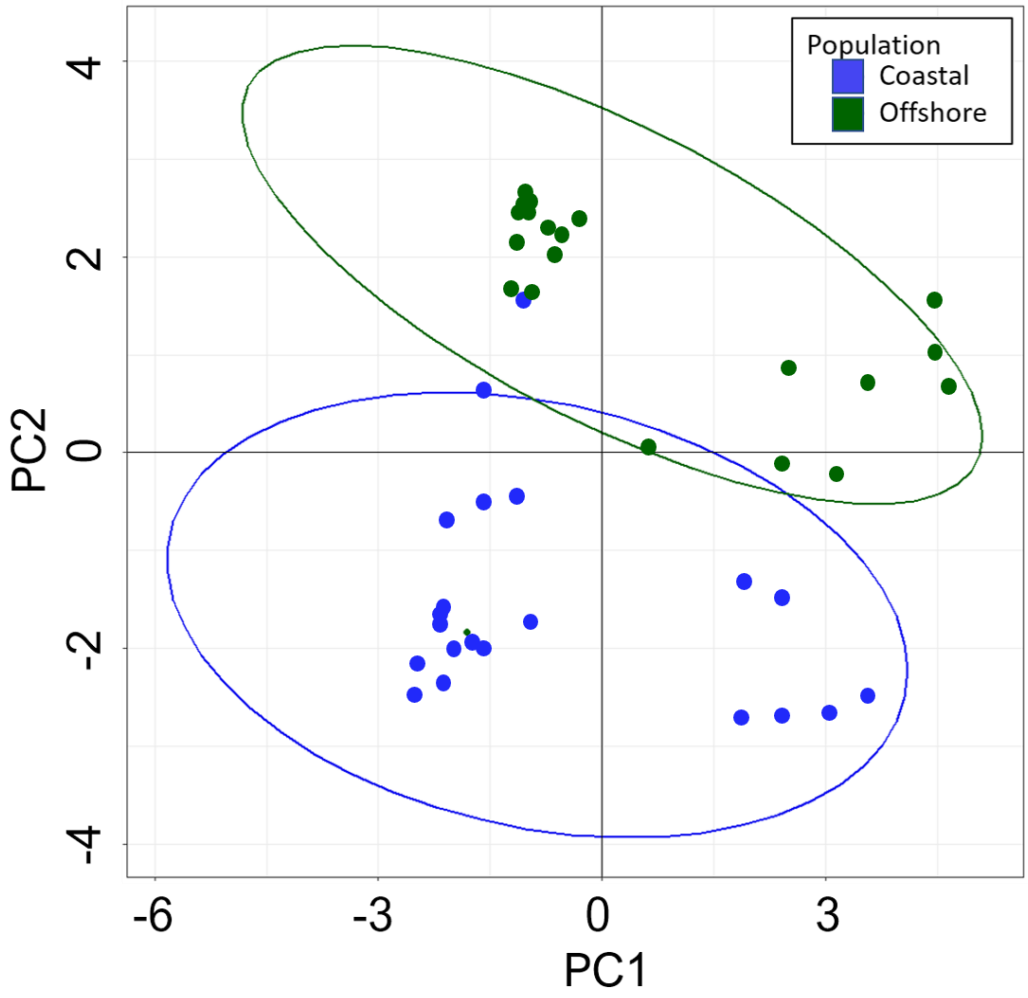

Figure 4.4: Principal Component Analysis of samples based on all loci showing the clear genetic distinction between Coastal and Offshore ecotypes. Note the apparent identification of a Coastal individual clustering with the Offshore ecotypes.

Table 4.1: Genetic distance values between WNA Coastal and Offshore ecotype populations. Values calculated in *R* using package *hierfstat.*

Comparison	F_{ST} (Pairwise) (Nei, 1987)	F_{ST} (Weir & Cockerham, 1984)	Da (Nei *et al*., 1983)
Coastal/Offshore	0.0141	0.0195	0.0162

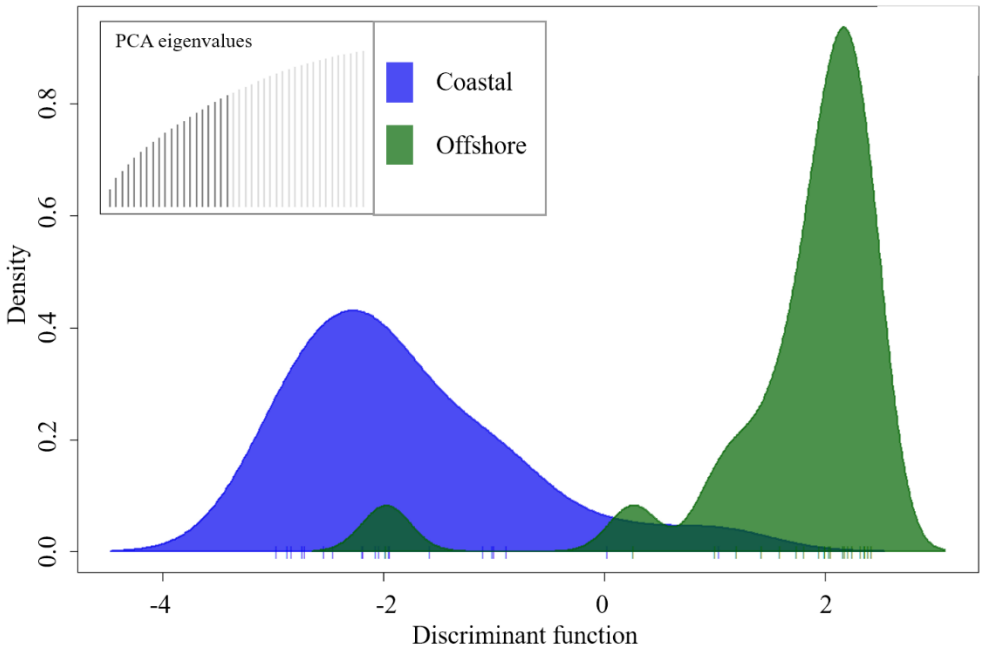

Figure 4.5: Discriminant Analysis of Principal Components plot for all loci of WNA *T. truncatus* showing the clear distinction between the two ecotypes.

Estimation of individual ancestries through the software *Admixture* revealed a similar story as previous analyses (Figure 4.7). There is clear genetic distinction between the two ecotypes but again there are several individuals (n=6) who show clear admixture, to varying levels, with the opposing ecotype. Four individuals also appear to be completely mis-assigned in their ecotypes, three of the four (P4, WNAP9, WNAP17) coming from the offshore ecotype but appearing to have complete coastal ecotype genetic ancestry and one (C5) where the reverse is true. The population probability assignment performed in *SambaR* (Figure 4.8) filtered out a number of admixed individuals due to higher QC filters but individuals of complete mis-assignment (n=2 – C5 and P4) and one individual with evidence of admixture, in this case a coastal individual (C3) showing admixture with the offshore ecotype, were retained.

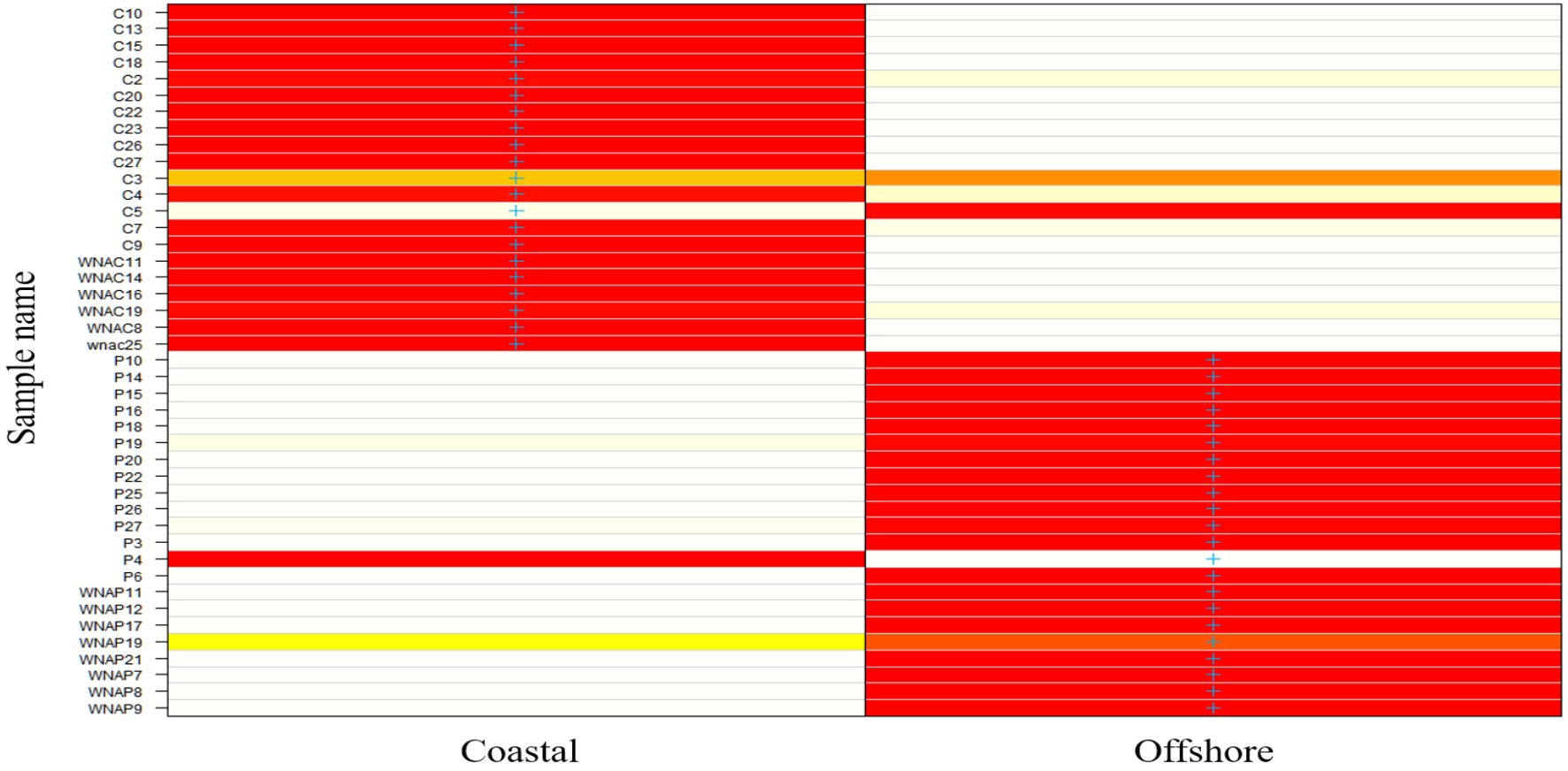

Figure 4.6: Assignment probability plot of individuals from DAPC analysis. The X-axis represents the possible assignment populations whereas the Y-axis represents the individual samples with representative sample names shown. Probabilities are represented by colour with red being high assignment probability, white being low and yellow being intermediate. With the exception of a few individuals, all individuals were assigned to their original presumed ecotype.

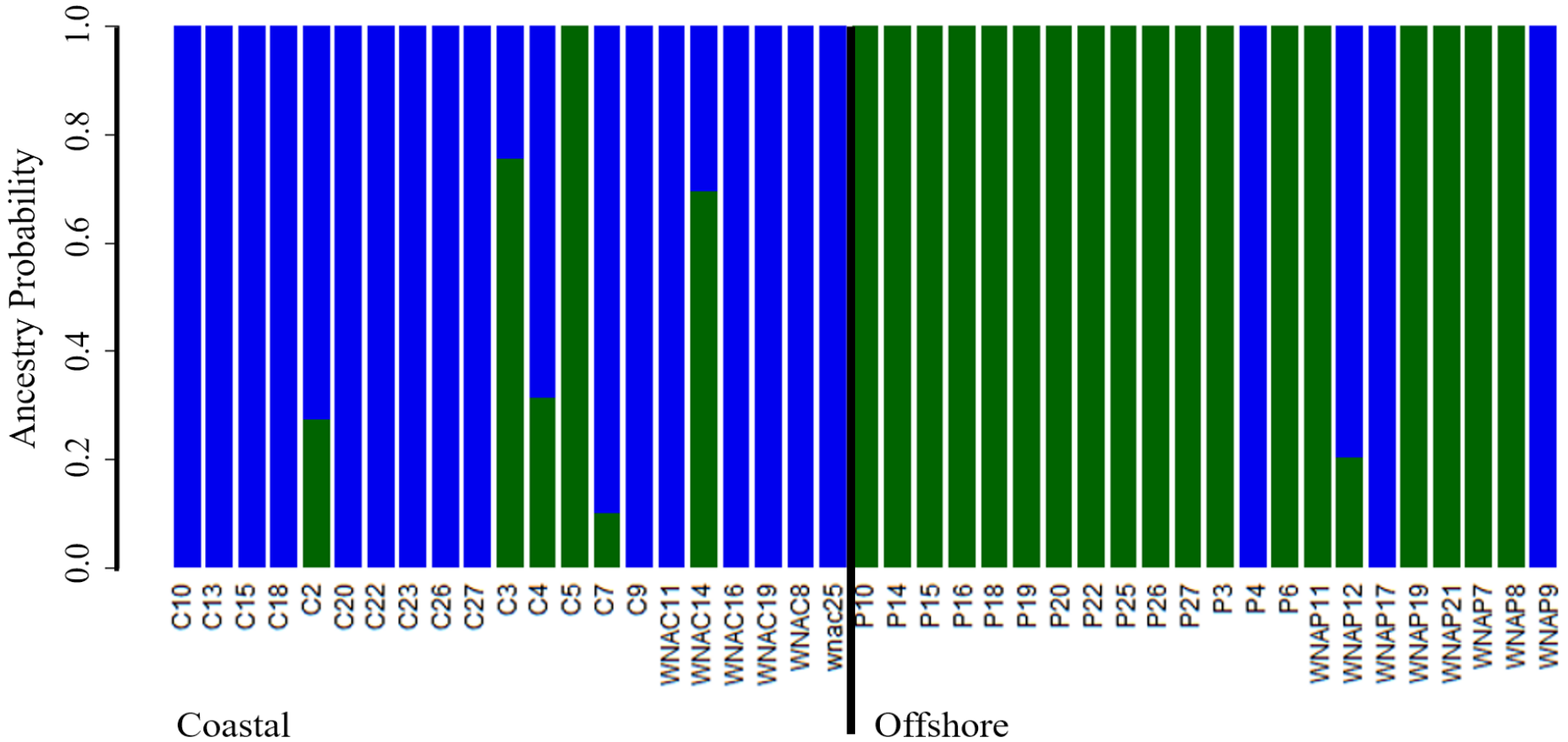

Figure 4.7: Estimated proportions of each individual's genome (admixture coefficient) that derives from hypothetical ancestral population 'K' (for K=2, Offshore and Coastal). Estimates developed in *Admixture* and visualised in *R*.

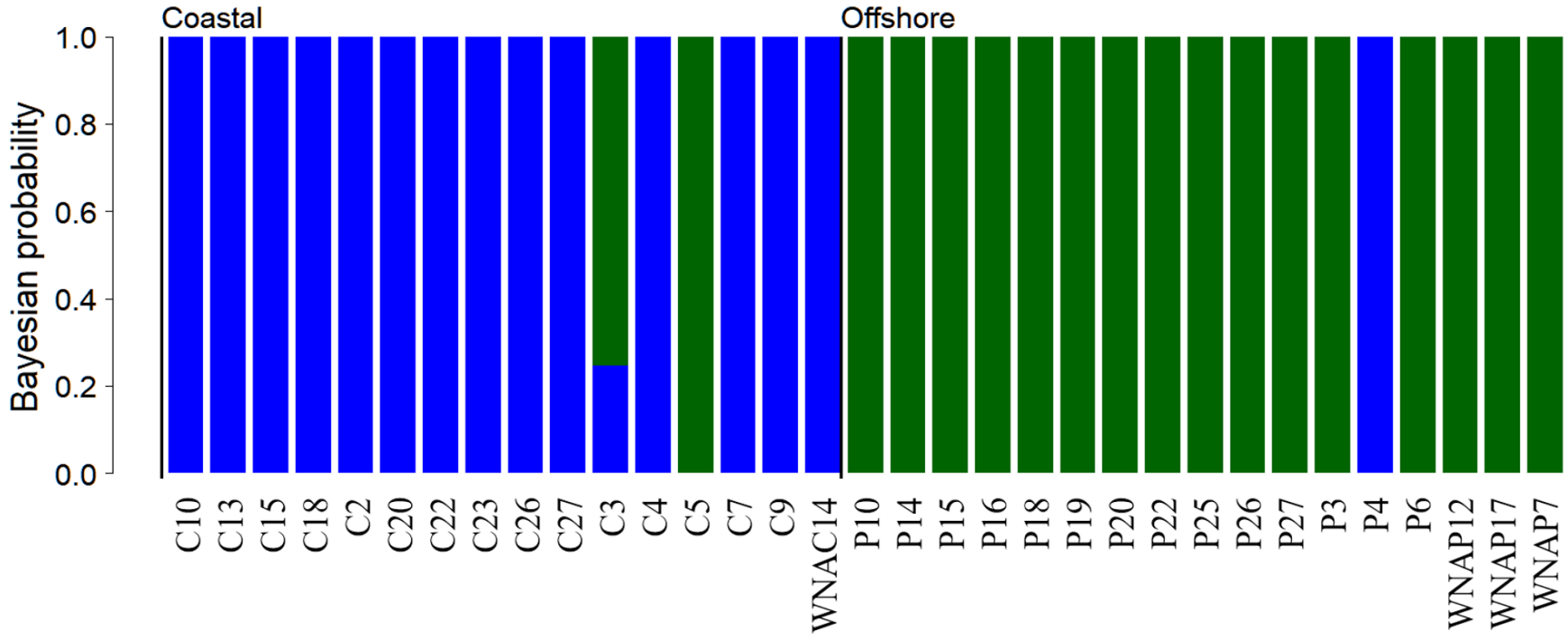

Figure 4.8: Probability barplot showing the probability that an individual belongs to either the Coastal or Offshore population given its observed genotype. Estimations performed in *SambaR*. Stringent quality filters reduced the number (n=33) of individuals included in this analysis.

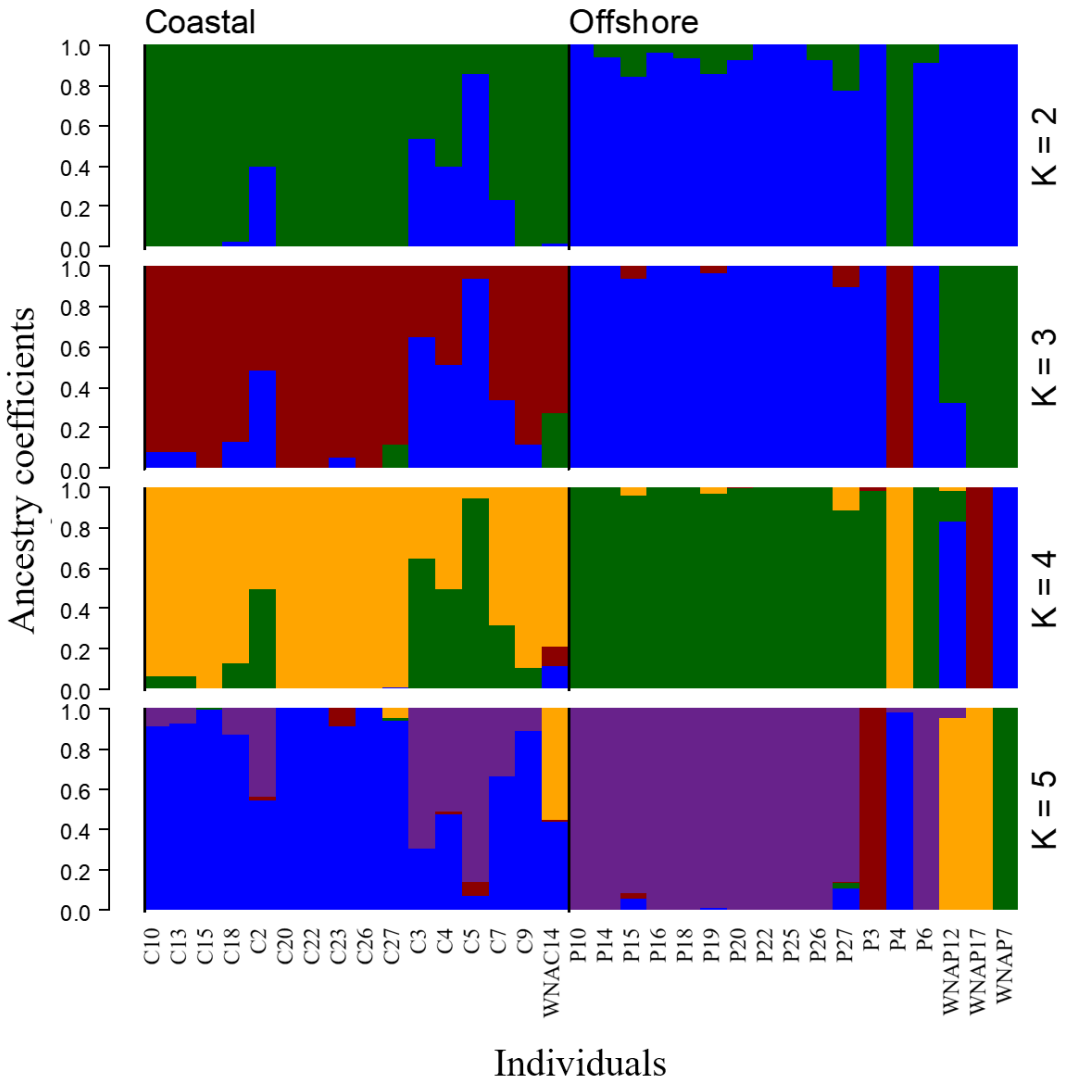

Figure 4.9: Estimated admixture coefficient for WNA individuals across all estimates of K from 2-5. Plot derived from the *snmf* function in the *R* package *LEA*.

Further investigations of population structure were conducted using the Landscape and Ecological Studies (*LEA*) package in *R* for values of *K* from 2 to 5 (Figure 4.9). K=3 was found to be the most supported hypothesis with two distinguishable ecotypes, with some offshore intrusion into coastal individuals and some offshore individuals forming their own group, presumably made up of ancestry significantly different from either ecotype but with high levels of intra-similarity.

Figure 4.10: Circosplot showing migration rates between the Offshore and Coastal populations as calculated by *BayesAss3-SNPs*. Figure migration values are found in Table 4.1.

Table 4.2: Matrix of inferred (posterior mean) migration rates per generation. Values in brackets represent migration as a proportion of population size. Migration rates are in the direction column to row, thus a slightly higher migration rate from the Offshore to the Coastal ecotype population was observed.

Population	Coastal	Offshore
Coastal	0.9485 (0.0299)	0.0515 (0.0299)
Offshore	0.0372 (0.0241)	0.9628 (0.0241)

Migration rates between the two ecotypes in the WNA are visualised in Figure 4.10. Migration rates calculated using *BayesAss3-SNPs* (summarised in Table 4.2) revealed that approximately 0.024% of the coastal population migrated to the offshore population per generation, just slightly less than the level of 0.029% of population per generation for the offshore to coastal direction. This analysis was repeated without samples that could have been mis-identified (Appendix VII) which showed an even stronger offshore to coastal migration rate. However, the more conservative results have been retained for this chapter.

4.3.2 Speciation and evolution in the genus Tursiops

Investigations into the evolution of the genus *Tursiops* began with calculation of D-statistics (ABBA-BABA testing) in *Dsuite* (Malinsky *et al*., 2019) to elucidate evidence of ancestral introgression. *Dsuite* tests for introgression between trios of populations (P1, P2 and P3) To interpret results a D-statistic of zero means no introgression has occurred whereas a value of one means an extreme event where there is no incomplete lineage sorting but introgression between P2 and P3.

A total of 84 trios were tested with a simple bifurcating tree inferred from recent literature (Moura *et al*., 2020). Of these trios, FDR adjusted p-values revealed 27 statistically significant D-statistic values, and of these 18 remained significant following a Bonferroni correction of the α-value to account for multiple testing (See Table 4.3). All 18 of these significant values derived from trios that contained populations of *T. aduncus* and *T. australis*. The highest statistically significant D-statistics were observed in trios where the populations South Africa and Burrunan occupied the second and third trio positions, suggesting the highest level of ancestral

gene flow in these lineages (Figure 4.11). The WNA Coastal and Offshore ecotypes displayed some evidence of ancestral introgression, and with some European populations following FDR adjustment but this did not remain significant following Bonferroni correction. That all D-statistic values for trios containing only various populations of *T. truncatus* were not significantly different from zero, following Bonferroni correction, could suggest that incomplete lineage sorting may be prevalent for these populations. Although substantial hybridisation and high levels of gene flow could present similar signals for *T. truncatus*, this study and others (see Moura *et al*., 2020) suggest that for many populations analysed here inter-population gene flow is substantially reduced (i.e. the Black Sea) such that this is unlikely to be the case.

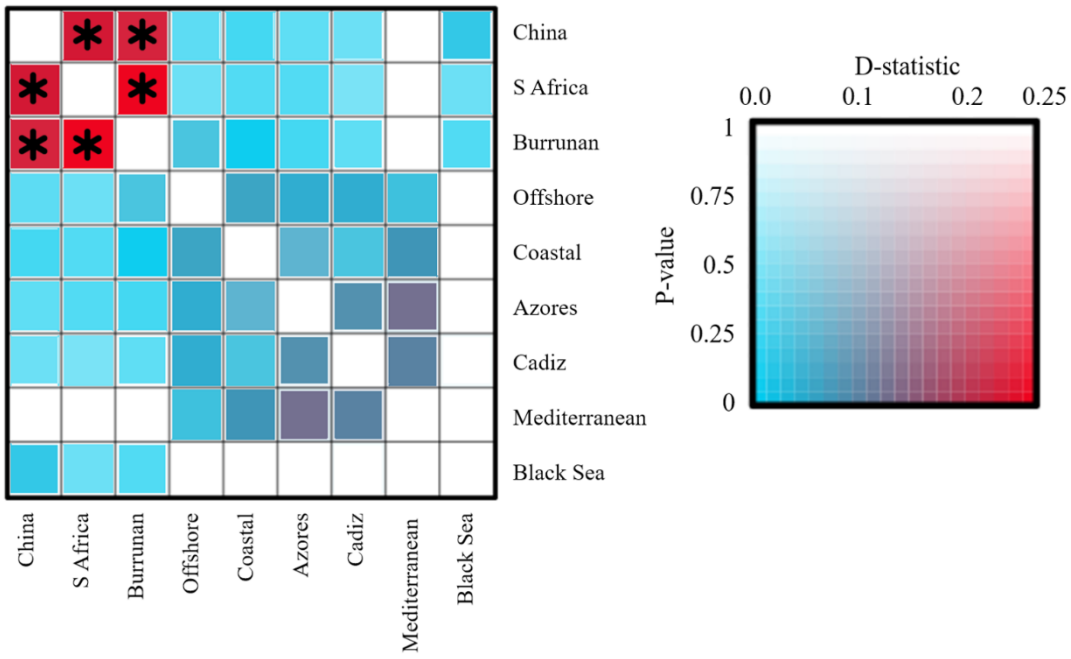

Figure 4.11: Heatmap showing results of D-statistics from *Dsuite*. Heatmap cell colour represents both D-statistic and its corresponding P-value for the maximum value of D between the two corresponding populations for any given population in first position of the trio. Significant results after FDR adjustment are marked by asterisks. Mediterranean and Black Sea populations have missing data due to their placement at the end of the tree data supplied to *Dsuite*.

Table 4.3: Calculated D-statistics for all possible population Trios (n=84), calculated by *Dsuite* (Malinsky 2019). *Dsuite* automatically arranges trios to give positive D-statistics and thus interpretation should be made based on introgression between P2 and P3. Q-values are FDR adjusted p-values with those in bold significant at the 0.05 level. Those in bold and italics are still significant following Bonferroni correction of the α-value ($\alpha_{corrected}$=0.000595).

P1	P2	P3	D-statistic	p-value	q-value	f_G
Azores	BlackSea	Burrunan	0.001	0.494	41.517	0.001
BlackSea	Cádiz	Azores	0.154	0.001	**0.001**	1.314
Azores	BlackSea	China	0.021	0.271	0.650	0.044
BlackSea	Azores	Coastal	0.096	0.002	**0.003**	0.638
BlackSea	Mediterranean	Azores	0.126	0.003	**0.005**	1.085
BlackSea	Azores	Offshore	0.079	0.010	**0.013**	0.746
BlackSea	Azores	SAfrica	0.013	0.413	2.314	0.018
Cádiz	Azores	Burrunan	0.003	0.417	2.696	0.005
Azores	Burrunan	China	0.269	0.000	***0.000***	0.901
Coastal	Azores	Burrunan	0.018	0.270	0.631	0.032
Mediterranean	Azores	Burrunan	0.033	0.129	0.221	0.059
Offshore	Azores	Burrunan	0.011	0.341	1.192	0.018
Azores	Burrunan	SAfrica	0.301	0.000	***0.000***	0.724
Azores	Cádiz	China	0.002	0.469	6.563	0.004
Azores	Cádiz	Coastal	0.004	0.417	2.502	0.028
Mediterranean	Cádiz	Azores	0.030	0.144	0.252	0.269
Azores	Cádiz	Offshore	0.014	0.308	0.923	0.331
Cádiz	Azores	SAfrica	0.010	0.266	0.588	0.017
Coastal	Azores	China	0.009	0.370	1.414	0.016
Mediterranean	Azores	China	0.023	0.176	0.336	0.044
Offshore	Azores	China	0.000	0.493	20.692	0.001
Azores	SAfrica	China	0.231	0.000	***0.000***	0.622
Mediterranean	Azores	Coastal	0.011	0.372	1.487	0.074
Coastal	Azores	Offshore	0.026	0.150	0.280	0.425
Coastal	Azores	SAfrica	0.018	0.335	1.126	0.030
Mediterranean	Azores	Offshore	0.023	0.220	0.462	0.366
Mediterranean	Azores	SAfrica	0.029	0.198	0.387	0.046
Offshore	Azores	SAfrica	0.011	0.290	0.786	0.017
Cádiz	BlackSea	Burrunan	0.003	0.467	5.601	0.006
BlackSea	Burrunan	China	0.251	0.000	***0.000***	0.560
Coastal	BlackSea	Burrunan	0.019	0.211	0.433	0.031
Mediterranean	BlackSea	Burrunan	0.033	0.048	0.072	0.058
Offshore	BlackSea	Burrunan	0.011	0.360	1.315	0.019
BlackSea	Burrunan	SAfrica	0.310	0.000	***0.000***	0.636
Cádiz	BlackSea	China	0.019	0.375	1.573	0.038
BlackSea	Cádiz	Coastal	0.100	0.001	**0.002**	0.572
BlackSea	Mediterranean	Cádiz	0.107	0.007	**0.010**	0.822
BlackSea	Cádiz	Offshore	0.093	0.002	**0.003**	0.780
BlackSea	Cádiz	SAfrica	0.003	0.480	10.088	0.004
Coastal	BlackSea	China	0.030	0.090	0.145	0.057

Mediterranean	BlackSea	China	0.044	0.050	0.076	0.082
Offshore	BlackSea	China	0.022	0.272	0.671	0.036
BlackSea	SAfrica	China	0.214	0.000	***0.000***	0.546
BlackSea	Mediterranean	Coastal	0.085	0.026	**0.038**	0.496
BlackSea	Coastal	Offshore	0.057	0.014	**0.020**	0.522
Coastal	BlackSea	SAfrica	0.005	0.462	4.851	0.008
BlackSea	Mediterranean	Offshore	0.059	0.054	0.084	0.497
Mediterranean	BlackSea	SAfrica	0.016	0.382	1.889	0.023
BlackSea	Offshore	SAfrica	0.002	0.486	13.612	0.003
Cádiz	Burrunan	China	0.265	0.000	***0.000***	0.663
Coastal	Cádiz	Burrunan	0.016	0.299	0.865	0.027
Mediterranean	Cádiz	Burrunan	0.030	0.145	0.265	0.053
Offshore	Cádiz	Burrunan	0.008	0.375	1.660	0.012
Cádiz	Burrunan	SAfrica	0.308	0.000	***0.000***	0.703
Coastal	Burrunan	China	0.274	0.000	***0.000***	0.689
Mediterranean	Burrunan	China	0.281	0.000	***0.000***	0.767
Offshore	Burrunan	China	0.268	0.000	***0.000***	0.647
SAfrica	Burrunan	China	0.043	0.145	0.259	0.128
Mediterranean	Coastal	Burrunan	0.015	0.127	0.214	0.027
Coastal	Offshore	Burrunan	0.008	0.331	1.068	0.013
Coastal	Burrunan	SAfrica	0.314	0.000	***0.000***	0.738
Mediterranean	Offshore	Burrunan	0.023	0.097	0.159	0.038
Mediterranean	Burrunan	SAfrica	0.319	0.000	***0.000***	0.690
Offshore	Burrunan	SAfrica	0.309	0.000	***0.000***	0.650
Coastal	Cádiz	China	0.011	0.407	2.137	0.020
Mediterranean	Cádiz	China	0.025	0.275	0.699	0.045
Offshore	Cádiz	China	0.003	0.440	3.699	0.004
Cádiz	SAfrica	China	0.228	0.000	***0.000***	0.612
Mediterranean	Cádiz	Coastal	0.015	0.283	0.743	0.093
Coastal	Cádiz	Offshore	0.040	0.041	0.060	0.540
Coastal	Cádiz	SAfrica	0.008	0.427	3.262	0.012
Mediterranean	Cádiz	Offshore	0.037	0.088	0.140	0.490
Mediterranean	Cádiz	SAfrica	0.019	0.295	0.826	0.030
Offshore	Cádiz	SAfrica	0.001	0.476	7.996	0.002
Mediterranean	Coastal	China	0.014	0.268	0.609	0.027
Coastal	Offshore	China	0.008	0.377	1.760	0.015
Coastal	SAfrica	China	0.237	0.000	***0.000***	0.576
Mediterranean	Offshore	China	0.022	0.207	0.414	0.043
Mediterranean	SAfrica	China	0.245	0.000	***0.000***	0.596
Offshore	SAfrica	China	0.231	0.000	***0.000***	0.529
Coastal	Mediterranean	Offshore	0.003	0.453	4.225	0.040
Mediterranean	Coastal	SAfrica	0.011	0.319	0.991	0.017
Coastal	Offshore	SAfrica	0.007	0.425	2.977	0.011
Mediterranean	Offshore	SAfrica	0.018	0.252	0.543	0.026

PSMC analyses demonstrate that until approximately two million years ago the effective population sizes (N_e) of *T. aduncus* and *T. truncatus* were concurrent in their trends, suggesting that they were in fact the same species at that time (Figure 4.12A). Then, after the Pliocene marine megafauna extinction and commencement of the quaternary glaciation, both species experienced a significant upward trend in N_e. However, this increase in N_e was greater for *T. aduncus* (reaching $N_e \approx 4x10^4$) than for *T. truncatus* (reaching $N_e \approx 3x10^4$). The increase in N_e ceased and indeed began to decline for both species approximately one million years ago and since that point has continued a general downward trend until the present day, albeit with occasional upward fluctuations (Figure 4.12C). Interpretations of N_e closer to the present day (<20kyr) should be treated with caution as inference is less robust (Li and Durbin, 2011). However, it can be concluded that present day N_e is certainly less than it has been in the past for both species. The pseudo-diploid analysis (h*PSMC*) (Figure 4.12B) revealed an N_e that tracked that of both *Tursiops* species up until around two million years ago before it diverged, rising to reach a trajectory towards infinity around one million years ago. It can therefore be inferred that time of divergence between the two species and the end of significant gene flow was around this time (≈1mya).

To infer ancestral divergence times and rates of gene flow I utilised Generalised Phylogenetic Coalescent Sampler (*G-PhoCS* (Gronau *et al*., 2012)), a Bayesian coalescent approach. Assessment of coalescence was completed using *Tracer* v1.6 (Rambaut *et al*., 2018) and showed that output estimates stabilised after approximately 60,000 iterations. Divergence times were calculated using *G-PhoCS* estimates of tau (τ) and calibrated with an estimated per generation mutation rate (μ) for cetaceans of $1.5x10^{-8}$ (Moura *et al*., 2014) and an average *Tursiops* generation time of 21.5 years (Taylor *et al*., 2007).

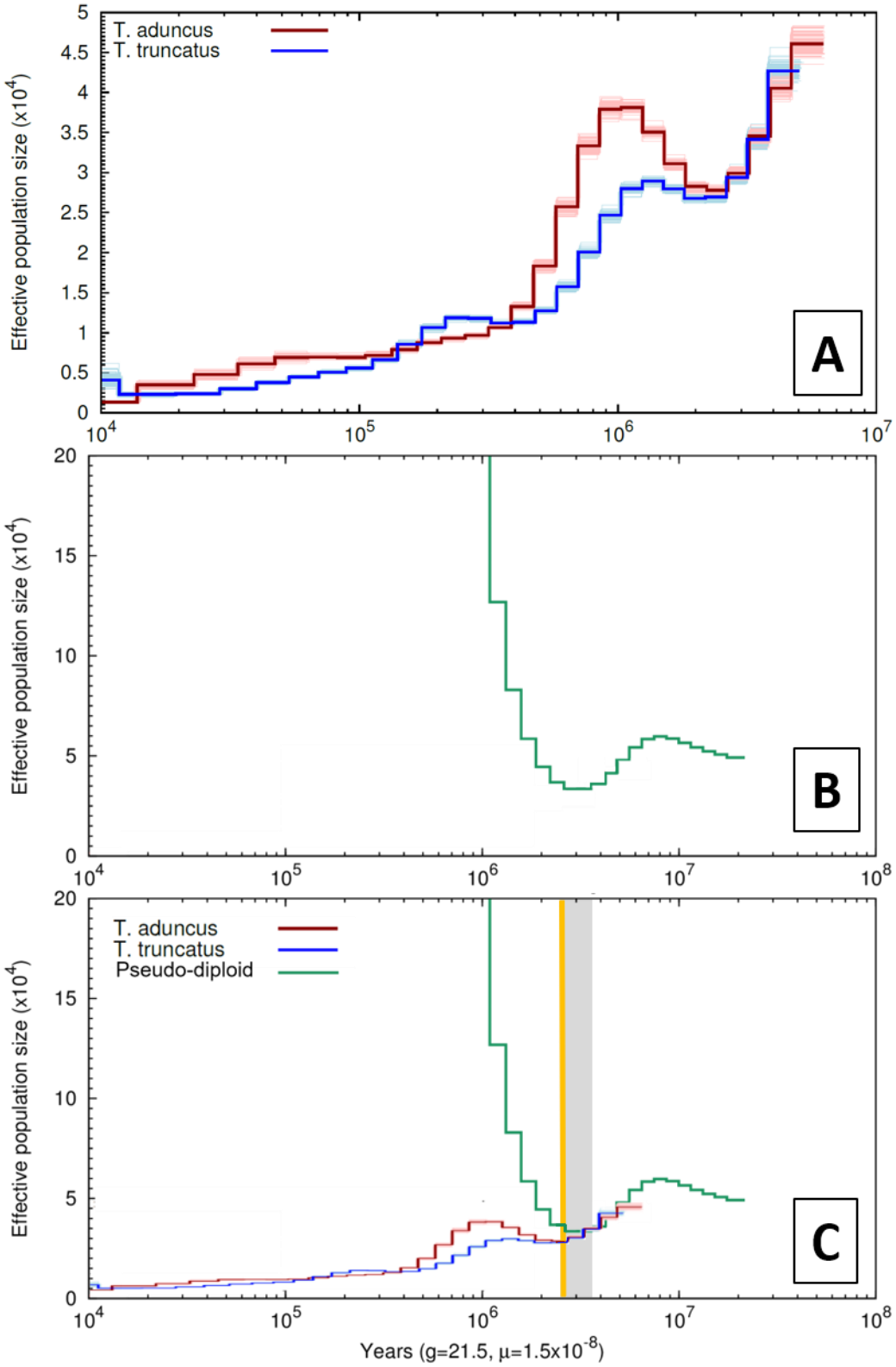

Figure 4.12: Demographic analysis of *Tursiops aduncus* and *Tursiops truncatus* genomes. A) shows *PSMC* analysis suggesting divergence beginning around 2 million years ago. B) depicts the pseudo-diploid, or pairwise-haploidized, *PSMC* (h*PSMC*) with divergence suggested to be around 1 million years ago. C) Overlay of both analyses. Grey-shaded vertical bar indicates the Pliocene marine megafauna extinction where 36% of genera were lost. Vertical gold line indicates the onset of the Quaternary glaciation and the lowering of sea levels.

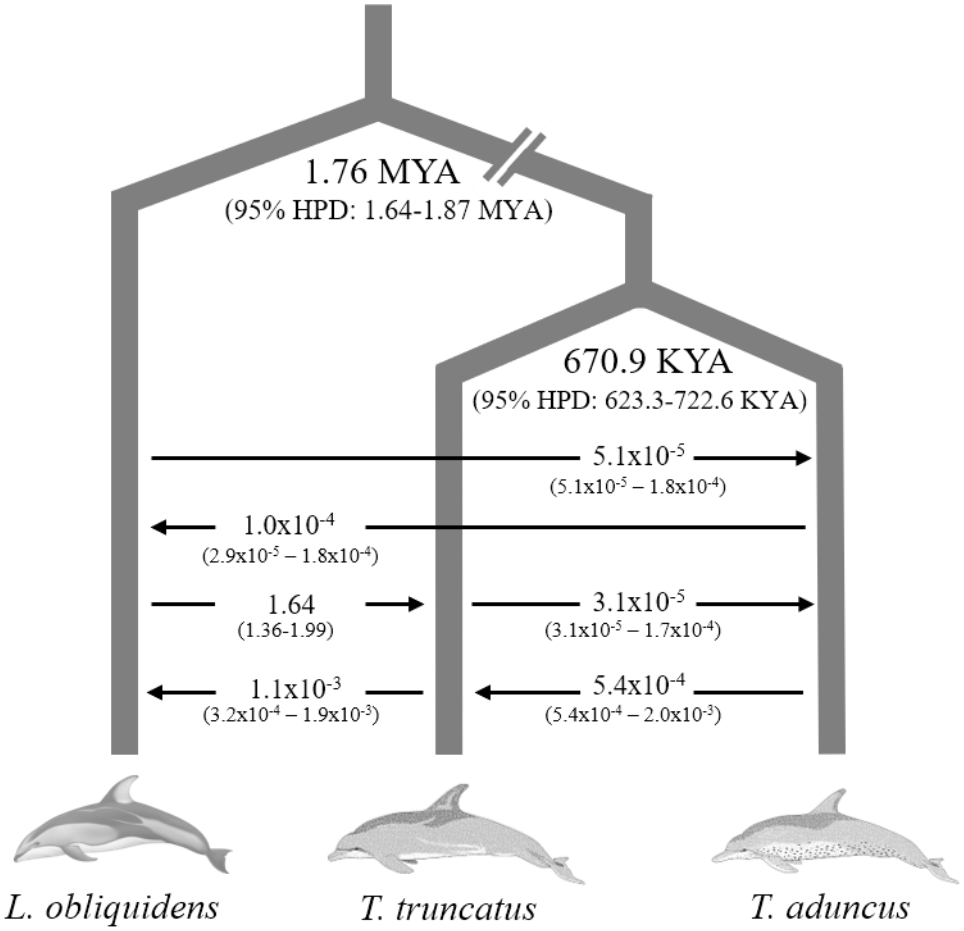

Figure 4.13: Demographic model of divergence time and migration rates inferred using *G-PhoCS*. Estimates of divergence time (in millions of years ago (MYA) or thousands of years ago (KYA)) are provided along the black dotted lines in bold level with each divergence node and 95% HPD intervals are provided in parentheses. Levels of migration are indicated by the migration bands which show directionality and figures indicate migrants per generation (with 95% HPD in parentheses).

Time of divergence between the *Tursiops* lineage and that which contained *Lagenorhynchus obliquidens* was estimated to be 1.76 million years before present with a 95% Highest Posterior Density (HPD) interval spanning the period 1.64 to 1.87 million years before present. The estimate divergence time between the *T. truncatus* and *T. aduncus* lineages was 670.9 thousand years before present, within a 95% HPD interval of 623.3 to 722.6 thousand years before present (Figure 4.13).

Migration bands were modelled in all directions for exploratory purposes. *G-PhoCS* infers the migration rate from population A to population B, given as $m_{A\text{-}B}$.

This can be converted to number of migrants per generation (M_{A-B}) using $M_{A-B} = m_{A-B} \times \theta_B$ where θ_B is the mutation rate for population B. Using this conversion, it was revealed that migration rates between *T. aduncus* and *T. truncatus* were relatively low in both directions (*T. aduncus* → *T. truncatus* 5.4×10^{-4} migrants per generation and *T. truncatus* → *T. aduncus* 3.1×10^{-5} migrants per generation), consistent with earlier ABBA-BABA statistics which also showed low levels of introgression between the two *Tursiops* lineages. Interestingly, *G-PhoCS* inferred the highest levels of gene flow from *L. obliquidens* to *T. truncatus* at 1.64 migrants per generation.

Unfortunately, *G-PhoCS* produced improbable estimates of effective population sizes (N_e) (e.g. for *T. truncatus* ≈ 3.6 million) with large 95% HPD intervals. These values should be treated with caution and are considered further in the discussion. The estimates of divergence between *T. aduncus* and *T. truncatus*, as derived from *PSMC* and *G-PhoCS* can now be placed within the wider context of evolution in the genus *Tursiops* (Figure 4.14).

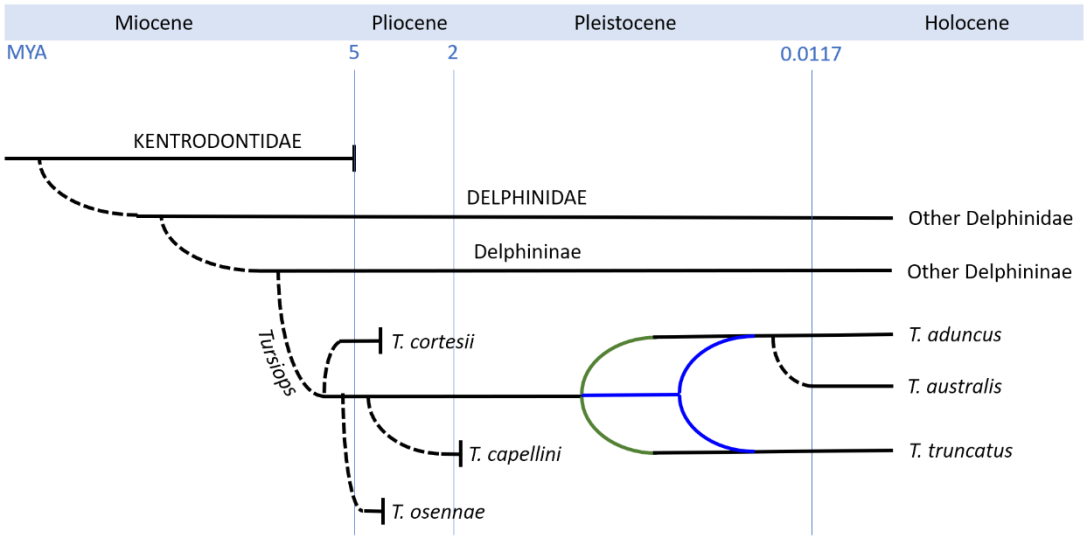

Figure 4.14: Schematic of the evolution of the genus *Tursiops* showing the new time-defined divisions of *T. aduncus* and *T. truncatus* in the mid-Pleistocene from *PSMC* (Green) and *G-PhoCS* (Blue). Dashed lines indicate presumed or estimated division times.

4.4 Discussion

The results of this chapter build on previous genetic studies (Fruet *et al*., 2017; Hoelzel *et al*., 1998; Louis *et al*., 2014; Lowther-Thieleking *et al*., 2015; Moura *et al*., 2020; Segura *et al*., 2006) and show clearly that differentiation between ecotypes in *Tursiops truncatus* is not just morphological (Costa *et al*., 2016; Félix *et al*., 2018; Perrin *et al*., 2011; Santillán *et al*., 2008; Simões-Lopes *et al*., 2019; Toledo, 2013), behavioural (Oudejans *et al*., 2015; Salinas-Zacarias, 2005) or trophic (Barros *et al*., 2010; Dıaz-Gamboa, 2003; Perrin *et al*., 2011; Segura *et al*., 2006), but that there is substantial genetic differentiation too.

Importantly however, although all analyses demonstrated the clear genetic differentiation between the offshore and coastal ecotypes of *T. truncatus* in the Western North Atlantic (WNA), as shown by Hoelzel *et al*. (1998), the high resolution advantage gained by employment of Next Generation Sequencing (NGS) technologies revealed low but important levels of gene flow between the ecotypes. Additionally, the identification of some individuals from a given ecotype appearing genetically to be from the other ecotype could suggest that there is either occasional social interaction or that individuals sometimes frequent the traditional geographic ranges of their opposing ecotype. Owing to the careful labelling and laboratory protocols employed in this study it is unlikely, though admittedly possible, that complete mis-identification of individuals has taken place. If this is indeed the case then this would have occurred at the point of stranding (i.e. during the 1980s).

If not misclassified then these individuals may be migrants. Migrants have been documented in a number of genetic studies (Fernández *et al*., 2011b; Fruet *et al*., 2014). *T. truncatus* have been known to travel over significant distances (O'Brien *et al*., 2009; Robinson *et al*., 2012) so the relatively short distance between these two

ecotypes is no barrier at all. The sex of these ecotype migrant individuals is currently unknown but in other regions *Tursiops* migrants have been found to show a sex bias. In *T. aduncus* males have been shown to be the principal dispersing sex (Möller and Beheregaray, 2004). In the Mediterranean, investigations in to gender dispersal has shown that, though not significant, migrants are more typically female (Gaspari *et al*., 2015a).

The movement of solitary *T. truncatus* away from established social groups is well documented (Dudzinksi *et al*., 1995; Eisfeld *et al*., 2010; Lockyer, 1978; Müller and Bossley, 2002). In these cases, the adult solitary *T. truncatus* is often active in evading interaction with new dolphin groups, a fact which would preclude gene flow between ecotypes. It could however, provide an explanation for the appearance of genetic 'coastal' *T. truncatus* in the geographic locations occupied by the offshore ecotype and *vice versa*. However, the occurrence of solitary dolphins among a population is relatively rare and by and large *T. truncatus* are gregarious, sociable animals. Although this could possibly explain a single occurrence it is unlikely that multiple solitary dolphins within this relatively modest sample size exist.

The coastal population of *T. truncatus* found off the east coast of the USA make a northwards migration during the spring, settling into a more northerly distribution throughout the summer, before migrating south again in the autumnal period (Barco *et al*., 1999; McLellan *et al*., 2002; Taylor *et al*., 2016). Although the data for date of collection of these samples are not available and exact migration pathways have not been studied in this region, it is possible that, like in other regions, *T. truncatus* utilise or avoid currents (dependent of direction of travel) to aid migration (Photopoulou *et al*., 2011) which could cause some coastal individuals to stray further

offshore than otherwise intended, thus presenting the geographic mixing seen in this study.

Whichever mechanism or process brought these individuals in to the geographic domain of the opposing ecotype it is clear that there has been resultant gene flow, as evidenced by individuals with clearly mixed ancestry. There was greater gene flow from the offshore to coastal ecotype populations and although it is unknown if this difference was significant there is now strong suggestion in the literature that in the western hemisphere the offshore populations of *T. truncatus* are likely the original source for many coastal populations (Moura *et al*., 2020, 2013). Future investigations may be able to demonstrate that the gene flow observed here is a microcosm of that wider pattern.

My assessment of D-statistics revealed no significant results, following Bonferroni adjustment, for comparisons of trios which included both the offshore and coastal ecotypes of the WNA which could indicate a lack of ancestral introgression between the two groups (significant D statistics do not indicate directionality). However, the results discussed previously indicate that gene flow between the ecotypes is detectable and not insubstantial. It is possible that incomplete lineage sorting is preventing clear interpretation here. Indeed, this would appear to be the case for all *T. truncatus* groups assessed and this is not without precedent as incomplete lineage sorting has been proposed for this species previously (Amaral *et al*., 2012; Gaspari *et al*., 2015b; Moura *et al*., 2013; Segura *et al*., 2006). However, existing phylogenies for the WNA populations have shown them to exhibit reciprocal monophyly (Hoelzel *et al*., 1998); indicating that the lineages have been separated for a significant amount of time, with little ancestral admixture, and that gene flow shown through *Admixture* could be more recent and an example of secondary contact.

The divergence time between *T. aduncus* and *T. truncatus*, estimated by *PSMC* analyses, is approximately one million years ago (Late Calabrian Stage, Pleistocene) but with commencement of differentiation beginning around two million years ago (Late Gelasian Stage, Pleistocene). This is in line with previous assessments by McGowen *et al*. (2009) who estimated a divergence time between 0.93 and 2.29 million years before present. It has been previously suggested that speciation within the *Tursiops* genus is likely driven by climate oscillations and the corresponding opening and closing of coastal habitat that this brings (Gray *et al*., 2018; Moura *et al*., 2013). It is thought that the common ancestor of these two species is likely to have been a coastal dwelling *Tursiops* species in either the Indian or Pacific Ocean regions (Moura *et al*., 2020). The initial commencement of quaternary glaciation, approximately 2.58 million years ago (Gibbard, 2015), may have forced this precursor species, at least in some areas, into more pelagic habitats where falls in sea level rise restricted access to continental shelf regions.

On first comparison it appears that there is a measurable difference between the estimated divergence time between *T. aduncus* and *T. truncatus* for *G-PhoCS* (670 KYA) and *PSMC* (1 MYA). This is in part due to the distinct models that each program utilises but also due to lack of confidence limits in *PSMC*. *PSMC* examines heterozygous site density within an individual genome sequence and does not directly infer divergence times, instead this is ascertained from divergence of estimated N_e traces when plotted on a time axis. However, as this estimate is without confidence limits and is effectively inferred by eye, there is scope for this estimate to be from a broad range and potentially overlapping with *G-PhoCS* estimates. By contrast, *G-PhoCS* assumes constant N_e for each lineage, thus preventing any gradual divergence in population sizes and divergence times are estimated only when separation is

complete, as well as coming with confidence limits which in this case gives the highest estimate of divergence as 722 KYA. Adopting a cautious interpretation and noting that for both analyses these data come only from single individuals, it is prudent to state that divergence likely occurred within the range of ≈620KYA to ≈1MYA.

Mean estimations of N_e in *G-PhoCS* provided high values for all lineages investigated, including 3.61 million for *T. truncatus* and 3.62 million for *T. aduncus*, and had relatively large HPD intervals. These values should be treated extremely cautiously, especially in the context of the presented *PSMC* analysis that estimated much lower values of ancestral N_e in *T. truncatus* (40-50,000 (published in Moura *et al*., 2020)). Such high values seem unlikely. *G-PhoCS* is known to have limited power to resolve demographic events in recent history (Gronau *et al*., 2011) and so recent reductions in population size may not be detected, as observed in other studies (Choi *et al*., 2017). However, *PSMC* analysis suggests this is not the case as N_e never appears to have reached the values suggested by *G-PhoCS*. Very high estimates of N_e (in the millions) from *G-PhoCS* have been published for other taxa (Campagna *et al*., 2015) where it has been observed that incomplete lineage sorting is likely the driver of these elevated values. Given the results of D-statistic analyses and known incomplete lineage sorting in *T. truncatus* (Amaral *et al*., 2012; Gaspari *et al*., 2015b; Moura *et al*., 2013; Segura *et al*., 2006) this seems a likely explanation. However, the overall trend of population size reduction from ancestral states to extant forms outputted from *G-PhoCS* fits with a wider phenomenon being observed across a broad array of cetacean taxa that shows a general decrease in cetacean abundance since the early Pleistocene (Warren *et al*., 2017; Moura *et al*., 2014; Árnason *et al*., 2018; Kishida, 2017).

Assuming a divergence time range for *T. aduncus* and *T. truncatus* of ≈620KYA to ≈1MYA, many estimates within this range would fall within the Donian glaciation (0.5-0.8MYA), one of the most extreme glaciation events of the quaternary (Bendixen *et al*., 2018). Although sea temperatures during the Donian glaciation were less affected than other glacial periods, the volume of ice that formed resulted in a huge sea level fall and subsequent loss of large amounts of shallow water habitat worldwide.

Cyclical climate events and in particular periods of glaciation or global cooling driving the evolution of Cetacea is not without precedent. The Delphinidae diverged from the Kentrodontidae during the Miocene, following which they began a rapid expansion and diversification such that within 3-4 million years all of the extant families were present (McGowen *et al*., 2009). It has been suggested that this rapid diversification was driven by a period of cooling in the Middle-Late Miocene (approximately 13-15 million years ago) (Fordyce and de Muizon, 2001), during which a significant expansion of the East Antarctic Ice Sheet would have impacted sea level, oceanic circulation and temperature gradients (Flower and Kennett, 1994). Given the precedent of climatic cooling influencing the emergence of Delphinidae and later Delphininae, it would then appear that a further climate cooling period in the Pliocene resulted in the rapid divergence and radiation of the genus *Tursiops* (Barnes, 1990; Whitmore, 1994). It is important to consider here that coincidence between species divergence times and climate events does not imply direct causal effect. It is more likely, considering the known physiology and adaptation potential of *Tursiops* spp., that climate events more readily affect prey population distributions and other biological factors (apart from the direct exclusion from shallow water habitats as a

result of sea level fall) and it is the impact of these shifts that provides a more causal mechanism for evolution in cetaceans (Hoelzel and Moura, 2015; Moura *et al*., 2014).

The most recent common ancestor (MRCA) of *T. aduncus* and *T. truncatus* is thought to have been a coastal inhabitant of Indo-Pacific origin (Moura *et al*., 2020, 2013). Evidence presented in this study, and in previous works (Moura *et al*., 2020), suggest that through a shift in prey resources or direct habitat exclusion an element of the MRCA population was forced offshore to form the *T. truncatus* lineage. In this scenario there is increased likelihood that the now pelagic *T. truncatus* would come in to more regular contact with other pelagic species than the coastal *T. aduncus*. Evidence of historical *T. truncatus* introgression with the pelagic Striped Dolphin *Stenella coeruleoalba* in the north Atlantic is reported by Moura *et al*. (2020) and in this study *G-PhoCS* suggests strong historical gene flow from the Pacific White Sided Dolphin *Lagenorhynchus obliquidens* to the *T. truncatus* lineage. Then, as *T. truncatus* began to occupy coastal niches, predominantly in the western hemisphere, the most likely candidate for introgression with the presently offshore *T. truncatus* becomes the parapatric coastal populations of *T. truncatus*. This would occur where habitat boundaries may overlap such as seen in the WNA in this study. This proposed scenario of historical environmental separation between *T. truncatus* and *T. aduncus* could explain the lack of introgression observed between the two lineages seen in this study, despite their known ability to produce fertile hybrid offspring (Gridley *et al*., 2018).

Successful occupation of a new environmental niche by a species is prerequisite on that niche being available in the first instance (Brockhurst *et al*., 2007). Immediately prior to the divergence of *T. truncatus* and *T. aduncus* the world's oceans experienced an extended period of loss of many large predatory species, including cetaceans, now called the Pliocene Marine Megafauna Extinction (PMME) (Pimiento

et al., 2017). The PMME occurred over a period of 1.4 million years (2.4-3.8 Ma) and saw a total loss of 36% of global marine megafauna species (covering seabirds, marine mammals, sea turtles and sharks). Of this loss, marine mammals experienced the greatest taxonomic loss with an estimated extinction of 55% of genera, of which 19% were thought to be found predominantly in offshore waters. This loss included several early species of *Tursiops* (Barnes, 1990). Combined with a loss of 9% of shark species, 60% of which were found offshore, and considering that offshore estimates are likely to be underrepresented due to differential fossil preservation/sampling bias, this represents a significant offshore niche vacancy.

In addition to an environmental niche being available for occupation, it is also required that at least part of the contemporary species is able to adapt to that newly available niche. It could be presumed that the divergence of *T. truncatus* and *T. aduncus* in the Australasian geographic area would be more likely than in any other possible geographic region owing to its increased relative coastline and bathymetric complexity. This increased complexity would facilitate greater local adaptation and thus genetic diversity in the precursor species. Local adaptation, even over relatively short distances, appears to be common within *Tursiops* spp. (Gaspari *et al*., 2015b; Gray *et al*., 2018). An increase in genetic diversity can increase species plasticity to adapt to new environments, in this case deeper offshore waters, before a complete genetic adjustment is made (Levin, 2010), thus increasing the likelihood of a successful new niche occupation.

It can now be concluded to accept hypothesis one as genetic differentiation between offshore and coastal ecotypes of *T. truncatus* has been shown to be clear, albeit with evidence of gene flow which also allows acceptance of hypothesis two. Hypothesis three shall remain neither proven nor disproven as whilst both *G-PhoCS*

and *PSMC* estimates of divergence are coincidental with the onset of major glaciation events there is neither enough confidence in the dating nor sufficient data to link cause and effect. The literature clearly supports the great influence that environmental factors have had on the evolution of cetaceans and whilst it is likely that glaciation onset was a driving factor in the divergence of *T. truncatus* and *T. aduncus*, further study is needed to reveal which event began this process and by what mechanism.

4.5 Conclusion

This chapter has confirmed that the offshore and coastal ecotypes of *T. truncatus* have clear genetic differentiation. Furthermore, the application of NGS technologies has provided the higher resolution needed to demonstrate clear gene flow between the ecotypes.

Gene flow between the early *T. aduncus* and *T. truncatus* lineages was likely sufficient that complete speciation took a significant period of time, as suggested by *PSMC* analysis. However, once *T. truncatus* occupied offshore waters there was very little gene flow between the two *Tursiops* species, with *T. truncatus* instead showing gene flow with other delphinid species. Historical admixture within the *Tursiops* genus was only significant within *T. aduncus* lineages and not between species. *T. truncatus* began to occupy coastal habitats through the Pleistocene, forming the coastal ecotype, with gene flow between the *T. truncatus* ecotypes continuing to the present day, likely resulting in incomplete lineage sorting in this species.

This chapter has improved estimation of divergence time between the two *Tursiops* species resulting in an estimated range of ≈620KYA to ≈1MYA. This period is concurrent with major environmental change, the Donian Glaciation, and it is possible that the reduction in sea level that this brought was a key driver in their speciation. Climate cycles have been suggested as a driver of cetacean evolution by previous studies and this chapter adds evidence to this hypothesis.

Further work should focus on narrowing the divergence time estimates between *T. truncatus* and *T. aduncus* as well as seeking to more explicitly interpret the mechanism by which climate cycles drive cetacean evolution, something which shall be considered in the final chapter of this thesis.

General Discussion

5.1 Summary of Key findings

This thesis set out to examine the population structure of two marine predators found in the Mediterranean and eastern Atlantic, the Bottlenose Dolphin *Tursiops truncatus* and the Yellowmouth Barracuda *Sphyraena viridensis* (summarised in Figure 5.1). The principal aims of doing so was to further understand how environmental features can influence marine predator population structure, either directly or through influence on distribution of prey resources.

Examination of *T. truncatus* revealed strong population differentiation across the area of study, including subtle structure not observed previously. The Black Sea population had the strongest genetic divergence from other groups and this study revealed, for the first time, evidence of gene flow between *T. truncatus* found in the Azores and those found around Sicily and Valencia. The observed population structure of *T. truncatus* correlated with some environmental features or variables; for example, salinity correlated significantly with genetic distance. There was correlation in stable isotope signature for $\delta^{15}N$, suggestive of a similar level of trophic feeding, between the Azorean and Sicilian *T. truncatus* populations, possibly caused by similar environments (as suggested by similar $\delta^{13}C$ values) dictating prey species availability.

Examination of *Sphyraena viridensis* found evidence for the presence of genetic population structure including some evidence for divergence between the Atlantic and Mediterranean. Within *S. viridensis* there are two clear haplogroups with an unequal geographic distribution. Across the species range there was evidence of

differential feeding by geography and several environmental factors, principally salinity, significantly explained at least part of the genetic variability. However, inferences were limited due to the limited genetic dataset.

When population structure of *S. viridensis* and *T. truncatus* was examined to consider the potential influence of oceanic fronts, no strong evidence was found to support the influence of the Siculo-Tunisian front on *T. truncatus*, despite previous suggestion to the contrary (e.g. Natoli *et al*. (2005)). However, the Almería-Oran front did appear to demarcate a transition zone between two populations of *T. truncatus* and further samples and examination may strengthen this interpretation. *S. viridensis* showed differentiation in haplogroup distribution between the Mediterranean and Atlantic though lack of available samples from the region of the Iberian Peninsula means the exact barrier to gene flow cannot be ascertained.

A secondary aim of this thesis was to examine potential gene flow between *T. truncatus* ecotypes and to understand potential environmental influence on speciation in this genus. High resolution examination of genetic differentiation between ecotypes (offshore and coastal) of *T. truncatus* in the WNA showed clear genetic differentiation but gene flow is still present. Incomplete Lineage Sorting (ILS) was suggested as a potential reason for lack of significant ancestral introgression seen between *T. truncatus* lineages compared to *T. aduncus*. This possibly indicates that *T. aduncus* is part of an older lineage than *T. truncatus*. There were multiple occurrences of correlation between major climate events and historical declines in N_e for several *T. truncatus* populations as well as with estimates of divergence time between *T. truncatus* and *T. aduncus*.

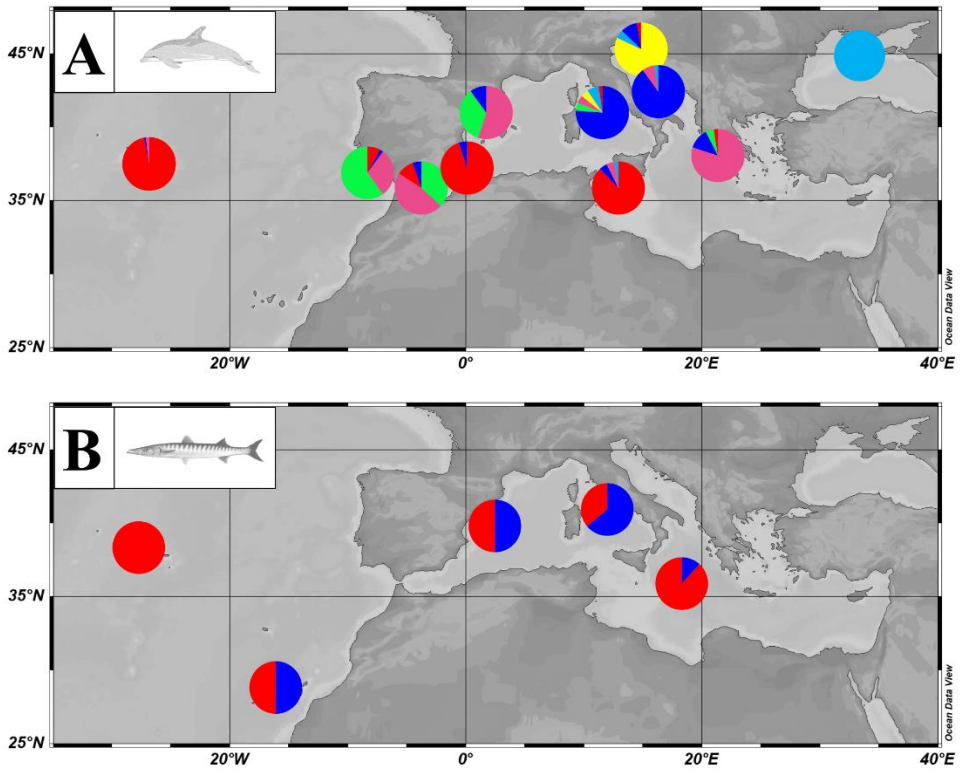

Figure 5.1: Population structure summary for (A) *Tursiops truncatus* showing Admixture population distributions and (B) *Sphyraena viridensis* haplogroup distributions.

5.2 Drivers of evolution in marine predators

The studies undertaken as part of this thesis have demonstrated that drivers of the formation of population structure, evolution and speciation in marine predators are varied, complex and often species specific. However, there are environmental factors examined herein that are proposed to drive evolution across a broad suite of marine taxa.

5.2.1 Environmental drivers of evolution in cetaceans

Examinations of population demography of *T. truncatus* (Sicily population – Chapter 2) and estimations of divergence between *T. aduncus* and *T. truncatus* (*G-PhoCS* and *PSMC* – Chapter 4) revealed correlation between the timing of major

events in the evolution of the genus or population constrictions, and the occurrence of cyclic climatic events. Various geological events, including glaciation and tectonic movements, have been correlated with major milestones in cetacean evolution previously (Fordyce and de Muizon, 2001; Steeman *et al*., 2009). The early Oligocene saw the emergence of early forms of mysticetes and odontocetes in the southern hemisphere and this radiation has been linked to the development of the psychrosphere (the cold deep-water layer of the oceans) which in turn increased upwelling and spiked ocean productivity (Fordyce, 1980). Such an increase in productivity would have created an upsurge in prey abundance and new niche opportunities; something which has been linked to increased cetacean diversity in other studies (Berger, 2007; Davies, 1963; Lipps and Mitchell, 1976; Marx and Uhen, 2010). Subsequent radiations in cetacean evolution are also linked with increasing heterogeneity in the ocean environment, and thus more niche opportunities, as the Oligocene progressed (Fordyce, 1992).

The aforementioned geological event-cetacean evolutionary step correlations typically derive from studies of the fossil record but there are now increasing numbers of molecular studies (this one now included) that support the hypothesis that environmental drivers have been key to cetacean evolution (Gaspari *et al*., 2015b; Moura *et al*., 2020, 2013; Steeman *et al*., 2009). Steeman *et al.* (2009) correlated periods of increased diversification rate in the cetacean phylogeny, as calculated via examination of nuclear and mtDNA markers, with periods of major restructuring in the world's oceans. Though this re-structuring principally refers to the closing or restriction of the world's major seaways (Tethys, Central American and Indo-Pacific), it would have been accompanied by increased complexity in ocean currents and major restructuring of prey distributions. This increase in complexity of ocean environments

created newly available ecological niches and molecular examination shows that cetaceans are adept at adaptive radiation, filling new niches quickly (Slater *et al*., 2010). These radiations include several extreme examples including multiple independent adaptations to riverine environments, allowing the species involved to avoid the tumultuous changes ongoing in the oceans (Cassens *et al*., 2000).

A major change in oceanic environments following periods of glaciation was the flooding of coastal habitat as sea levels rose (Lobo *et al*., 2001). This would have provided additional physical niches, in terms of shallow water coastal habitat, that provided opportunity for diversification beyond just the aforementioned newly available trophic niches (Steeman *et al*., 2009). Molecular evidence for past population expansions have been observed previously for coastal cetaceans, including *T. truncatus,* (Amaral *et al*., 2007; Banguera-Hinestroza *et al*., 2010; Moura *et al*., 2013; Natoli *et al*., 2004) and this could potentially be explained by the expansion in to these new environments. This process is likely key in the formation of *T. truncatus* ecotypes worldwide, as well as the colonisation of coastal habitats by pelagic populations (Gaspari *et al*., 2015b).

That sea temperatures should be a driver of evolution in cetaceans may at first seem at odds with their warm-blooded physiology and global distribution. However, prey resources may be more thermally restricted and those cetacean taxa that specialise may be subsequently geographically limited, with forced movement and adaptation as climatic cycles shift marine temperature distributions. Davies (1963) proposed that various odontocetes that had antitropical distributions, for example beaked whales Ziphiidae, were restricted in their movements between north and south populations by the warmer tropical waters of the equator. Davies (1963) went on to suggest that reduction in the barrier presented by tropical waters would have occurred during

Pleistocene glaciation events which in turn would have allowed the two populations to mix. These ideas have been tested in studies of *Lagenorhynchus* species that have antitropical distributions in the Pacific Ocean; whereby examination of nuclear and mtDNA markers revealed that the present genetic structure likely came about due to cyclical weakening of the tropical water barrier (due to cyclical glaciation events) with substantial population mixing occurring during each event (Cipriano, 1997; Hare *et al*., 2002). Beyond mere distribution, cetacean diversity has been linked to temperature dependent ocean productivity, both in the past (Marx and Uhen, 2010) and the present day (Whitehead *et al*., 2008). With the onset of current rapid climate change it is likely that cetaceans will be affected by sea temperature factors in the future too (Evans *et al*., 2010).

The correlation between water temperature and genetic population structure in cetaceans is, as aforementioned, likely attributable to temperature implications for prey resources rather than a direct effect on the cetacean taxa themselves. Studies have shown that prey species can influence population structure in cetaceans, through either intraspecies prey specialisation (Hoelzel *et al*., 2007) or dispersed and patchy prey distributions and consequent isolation by distance between predator populations (Amaral *et al*., 2012). For some whale species, socially reinforced fidelity for specific prey hunting grounds can add to this effect (Palsbøll *et al*., 1995; Viricel *et al*., 2016). Within this study no evidence of isolation by distance was shown and it is likely that within *T. truncatus* it is the intraspecific prey speciality mechanism, often implemented through ecotype formation, that is at play here. This thesis has demonstrated a strong correlation between population genetics, trophic feeding level and shared environmental features and even when one ecotype comes in to close proximity with another, such as in the Almería-Oran front region there remains strong

genetic distinction, akin to what is commonly observed in *Orcinus orca* (Moura *et al*., 2015; Pilot *et al*., 2010).

With the advance of modern molecular techniques, it is now possible to examine active or current adaptation through the detection of loci under selection and potentially genes linked to loci locations. Gene ontology studies on *Tursiops* spp. have revealed an abundance of target genes under positive selection that correspond to physiological adaptation to the aquatic environment (e.g. Nery *et al*., 2013). With the increasing accessibility of high-resolution genetic data, future gene ontology studies should examine individual populations to elucidate signals of selection that could be interpreted as being driven by differential environments.

5.2.2 Other possible drivers of evolution in cetaceans

This thesis revealed a correlation between the population structure of *T. truncatus* in the Mediterranean and eastern Atlantic and both social and acoustic traits for the same populations reported in other studies (La Manna *et al*., 2017; Papale *et al*., 2017, 2014). Social structure, and in particular cultural reinforcement of lineages, has been implicated as a possible driver of population structure formation and evolution in cetaceans before (Costa-Urrutia *et al*., 2012; Mesnick, 2001; Van Cise, 2017), including within *Tursiops* (Diaz-Aguirre *et al*., 2019).

Further work is needed to investigate this potential driver of evolution in the Mediterranean/Atlantic populations and future studies should seek to combine genetic data with acoustic and social datasets of which a number exist for this region (e.g. Carnabuci *et al*., 2009; La Manna *et al*., 2017; López, 2011).

5.2.3 Evolutionary drivers in teleosts

As with cetaceans, there is strong evidence for the influence of glaciation on the formation of population structure in teleosts (Hickerson and Ross, 2001; Solbakken *et al*., 2017; Wilson, 2006). The impact of glaciation on population structure and evolution in teleosts is complex and there is a great deal of variation between taxa. Some of this variation is due to the diversity of physiology, life history and other traits in teleosts. However, ecological similarity between species does not necessarily predict the influence that climatic cycles have on population structure (Haney *et al*., 2009).

The majority of studies on the influence of glaciation on teleost population structure and evolution focus on coastal or estuarine fish (e.g. Haney *et al*., 2009; Hickerson and Ross, 2001; Wilson, 2006) where it is easy to understand that restriction in access to habitat by sea level changes can have an impact. Equally, the restriction of a coastal species to isolated refugia will clearly limit gene flow and lead to population differentiation. By contrast, species that have much greater potential for larval dispersal and are less reliant on coastal habitat (such as *S. viridensis*) tend to be influenced in their population structure by glaciation only at a much broader geographical scale, if at all. For example, Canino *et al*. (2010) examined the impact of glaciation on the genetic population structure of Pacific Cod *Gadus macrocephalus* and found that, unlike in more coastal species, there was no evidence of local population differentiation because *G. macrocephalus* was not restricted to coastal glacial refugia. Instead the authors found evidence of ocean-basin scale differentiation likely due to glaciation acting as a barrier to gene flow between east and west lineages of the species, with the present-day gene flow being a result of secondary contact.

A further driver of evolution in teleosts that is of reduced importance in cetaceans, being largely top predators except when sympatric with *O. orca*, is adaptation to predation pressure. The presence of predators has been found to be the major driver of ecological differentiation in teleosts (Heinen *et al*., 2013; Langerhans *et al*., 2004), with sustained ecological differentiation likely being reflected in genetics where such studies are carried out. In *S. viridensis* predation pressure is likely to be highest during their larval and juvenile life stages, reducing as body size increases as seen in other teleosts (Gibson *et al*., 2002). Unfortunately, no predation pressure data are available for this species. Consequently, no inference can be drawn on the potential for this to be a factor in influencing the observed population structure but it may be an avenue for future research.

Many examples of teleosts displaying high levels of population structure derive from those species that are strongly associated with a specific habitat, such as coral reefs, and the patchiness of these habitats leads to Isolation By Environment (IBE) (Bay *et al*., 2008; Froukh and Kochzius, 2007; Nanninga *et al*., 2014; Ovenden *et al*., 2004; Salas *et al*., 2019). Often a key limiting factor for gene flow in teleosts is dispersal potential, with limited dispersal leading to Isolation By Distance (IBD) (Planes and Fauvelot, 2002; Purcell *et al*., 2009; Saenz-Agudelo *et al*., 2015). Although *S. viridensis* is commonly observed feeding around reefs (Barreiros *et al*., 2002; Cresson *et al*., 2014), it, like other barracudas (Daly-Engel *et al*., 2012; de Sylva, 1963), is not likely an obligate reef predator, with capacity for pelagic movements and thus high mature dispersal capacity in addition to its larval dispersal potential. When this hypothesis is considered in the context of other large predatory fish with high dispersal potential and non-specific habitat requirements, I can draw a general pattern of low observable fine-scale population structure with genetic differentiation more

likely at an ocean-basin scale, if present at all (Albaina *et al*., 2013; Boustany *et al*., 2008; Ely *et al*., 2005; Garber *et al*., 2005).

5.3 The influence of environmental boundaries

A fundamental aim of this thesis was to investigate the potential for oceanic fronts to act as a barrier to gene flow in marine predators, and to thus act as a driver for the formation of population structure. The introduction of this thesis (Chapter 1) laid out the pre-existing literature that considered the influence of either the Almería-Oran Front (AOF) or the Siculo-Tunisian Front (STF) on population structure across a wide range of taxa within the Mediterranean Sea (Table 1.1) and in so doing revealed huge variation in their impact, even within taxonomic groups. Furthermore, the mechanisms by which frontal systems influence population structure remain unclear. For example, for those species which have a larval stage as part of their life history it has been hypothesised that larval retention by frontal currents could be enough to create some of the divergence between populations observed; but even a small amount of larval leakage or adult migration has been shown to be enough to counteract this (Naciri *et al*., 1999). And yet despite this potential larval leakage, there are a growing number of examples in the literature where population structure studies highlight a correlation between population boundaries and frontal systems, including either the AOF or STF (Bourret *et al*., 2007; Cimmaruta *et al*., 2005; Gaspari *et al*., 2007; Natoli *et al*., 2008, 2005; Zardoya *et al*., 2004).

A principal problem for many of the studies highlighted in Table 1.1 is that most did not set out to purposefully examine the influence of ocean fronts on population structure, with a few exceptions (Galarza *et al*., 2009), it was merely a post-

hoc correlation between population boundaries and frontal regions. As such, these studies frequently suffer from a paucity in samples in key areas to allow confident inference. Despite the targeted nature of this study, the difficulty in obtaining samples of highly mobile top predators resulted in a similar disadvantage. However, excluding the offshore populations of *T. truncatus* (Sicily-Azores), this study revealed an east-west divergence in coastal *T. truncatus* in the Mediterranean. Unlike previous studies that identified this pattern and placed the line of divergence incident with the STF (Natoli *et al*., 2005), this higher resolution study placed it roughly in line with the Corsica-Sardinia line, something which has been observed for Albacore Tuna *Thunnus alalonga* (Davies *et al*., 2011; Montes *et al*., 2012) and Gilthead Seabream *Sparus aurata* (De Innocentiis *et al*., 2004).

The STF is a relatively weak and temporally unstable frontal system (Manzella, 1994) and it is perhaps more accurate to say it represents a steep gradient between the east and west Mediterranean basins rather than a sharp transition. For those, typically lower trophic and less mobile species where the STF appears to have acted as a barrier to gene flow (see Table 1.1), it is likely this represents local adaptation to prevailing environmental conditions and environmental selection against migrants. Many of those species that appear not to have population boundaries correlating to the STF are conspicuous in their homeothermic or endothermic physiologies (e.g. Bluefin Tuna *Thunnus thynnus* (Riccioni *et al*., 2013), Swordfish *Xiphias gladius* (Pujolar *et al*., 2002) Sperm Whale *Physeter macrocephalus* (Drouot *et al*., 2004) and Bottlenose Dolphin *Tursiops truncatus* (this study)) leading to the possible interpretation that factors other than water temperature are more important when it comes to formation of population structure, where present. For *T. thynnus*, water currents and asymmetrical larval transportation has been implicated (Carlsson

et al., 2004) though this does appear to run counter to the findings of Naciri *et al*. (1999) who suggested that even a low level of larval leakage from oceanographic features should be enough to counteract their impact as an agent of population division, over time.

This thesis reported a significant level of population differentiation between Adriatic and Tyrrhenian populations of *S. viridensis* and although speculative, this may be as a result of STF influence (alternative interpretations could include IBD as the line of travel around the Italian peninsula is considerable); but caution in this interpretation should be taken as low sample sizes and paucity of samples around Sicily mean hard conclusions cannot be drawn. Certainly, this is a question that should be revisited by the scientific community as greater sample numbers become available.

The Almería-Oran Front (AOF) is considerably different to the STF in that it is temporally stable and represents a sharp cline in environmental variables. Whilst the AOF may influence the population structure in *S. viridensis* this remains unclear due to lack of samples in this region. However, the AOF did appear to form a boundary line between the offshore associated Valencia *T. truncatus* and the coastally associated individuals of the Alborán Sea. This delineation could be due to differential feeding between ecotypes, as explored previously in this thesis, but isotopic or stomach contents analysis data are currently lacking for *T. truncatus* in the Alborán Sea. An alternative interpretation, especially in such a socially complex species, could be that *T. truncatus* use strong environmental boundaries, such as the AOF, as demarcations for territorial or social boundaries as seen in numerous other species (Eason *et al*., 1999; Heap *et al*., 2012). However, this is somewhat unlikely as although *T. truncatus* have been observed performing territorial type behaviours (Félix, 2001) they are generally accepted as being a non-territorial species (King *et al*., 2014).

5.4 Recommendations for future research

Whilst this study has substantially contributed to our understanding of contemporary populations of *T. truncatus* and *S. viridensis* in the Mediterranean and eastern Atlantic, and the evolutionary history of the genus *Tursiops* it has also presented many questions and indicated possibilities for future research.

A fundamental challenge for this study lay in obtaining samples that provided sufficient coverage of all geographic areas of interest. This is a common problem in examinations of marine predators where sample collection can typically require expensive sea time, equipment and niche training. To meet this challenge in the future research institutions should endeavour to be more collaborative with greater accessibility and sharing of samples; a willingness which was not always shown during the course of this study. Sample sharing schemes are becoming increasingly common, such as Shark Share Global which is now hosted on the sample sharing platform Otlet (Otlet, 2019), and it is recommended that all researchers contribute to such schemes with cetacean samples whenever possible. In addition, this study entirely lacked samples (for both species) from the north coast of Africa. Access to such samples could be crucially important in developing our understanding of population structure for both species; so whenever it is safe to do so, future efforts should be made to undertake or support locally collaborative research expeditions to obtain such samples. As more samples become available re-examination of ocean front influence on population structure formation could be a worthwhile endeavour.

In Chapter 2 I revealed evidence for an Azores-Valencia-Sicily metapopulation in *T. truncatus* that appeared to correlate with published patterns of social and acoustic data for this species. Future work should seek to obtain standardised social and acoustic data from across the study region and examine for

correlation with available genetic data. It is distinctly possible that social drivers, as well as environmental ones, play a significant role in the development of population structure in *T. truncatus*.

Population structure in *S. viridensis* was examined using mtDNA but a re-examination using a higher resolution dataset should be utilised in future research. Much groundwork has been laid in the development of a bait-capture microsatellite library (see Appendix IV for details) and this could be a route of future examination. Alternatively, the development of a SNP dataset via ddRADseq or similar protocol, as was utilised for *T. truncatus* in this study, may be able to reveal further population structure detail that is currently hidden. The utilisation of bi-parental markers such as SNPs or microsatellites will reveal a population structure that reflects migration of both males and females, which is not present in this study.

Finally, a major review of the fossil record for the genus *Tursiops* should be undertaken. The last review was undertaken thirty years ago (Barnes, 1990) when molecular phylogenetic techniques, and our understanding, were in their relative infancy. Whilst modern molecular studies (i.e. Moura *et al*., 2020) do make use of the best available fossil calibrations, the recent palaeontological literature for *Tursiops* spp. is relatively disparate A modern review would provide an extremely valuable synthesis and no doubt reveal avenues for future palaeontological research.

5.5 Concluding remarks

This study revealed complex fine-scale population structure in *Tursiops truncatus* in the Mediterranean and across the eastern Atlantic. Although the higher resolution data in this thesis have revealed some boundary shifts and subtle population structure not previously shown, the results are consistent with the high levels of localised population differentiation displayed by this genus (Dowling and Brown, 1993; Gaspari *et al*., 2015b; Gray *et al*., 2018; Moura *et al*., 2013; Natoli *et al*., 2005, 2004; Segura *et al*., 2006; Sellas *et al*., 2005; Viaud-Martinez *et al*., 2008). *T. truncatus* is well documented for establishing ecotypes (Fahlman *et al*., 2018a; Fruet *et al*., 2017; Perrin *et al*., 2011; Rossbach and Herzing, 1999; Segura *et al*., 2006; Torres *et al*., 2003) and the results in this thesis provide some evidence for ecotype presence in the Mediterranean and eastern Atlantic, which builds on previous work (Louis *et al*., 2014); as well as showing that genetic differentiation between ecotypes is strong, in agreement with previous examinations (Hoelzel *et al*., 1998; Lowther-Thieleking *et al*., 2015; Segura *et al*., 2006).

There have been numerous reports of long-term declines in cetacean populations across a wide range of taxa in an as of yet unexplained phenomenon (Warren *et al*., 2017; Moura *et al*., 2014; Árnason *et al*., 2018; Kishida, 2017) and examinations of *T. truncatus* population demographies in this study are consistent with this trend.

Investigations in to the divergence time of *T. truncatus* and *T. aduncus* revealed time estimates coinciding with periods of rapid environmental changes, namely the onset of glaciation. Glaciation events typically reduce available shallow water habitat through sea level fall and in so doing could provide the mechanism for divergence by forcing both prey resources and some populations of the coastal

dwelling *Tursiops* ancestor offshore. Many studies have now described how evolutionary steps in cetaceans have been correlated with major environmental change (Barnes, 1990; Berger, 2007; Davies, 1963; Fordyce, 1980; Fordyce and de Muizon, 2001; Gray *et al*., 2018; Lipps and Mitchell, 1976; McGowen *et al*., 2009; Moura *et al*., 2013; Whitmore, 1994) with the mechanism for such changes often being linked to changes in ocean productivity or prey resource distribution (Davies, 1963; Fordyce, 1980; A. R. Hoelzel, 1998; Moura *et al*., 2020).

Although there is some evidence presented by this thesis that ocean frontal systems coincide with population boundaries of *T. truncatus* it is clear that the formation of population structure in this species is complex and it is likely that it is not only environmental drivers at play. The observed population structure in *S. viridensis* suggests that ocean currents are likely important in the distribution of haplotypes. However, one thing that this thesis has clearly demonstrated is that making generalisations on the drivers of population structure formation across taxa is difficult. It is clear that environmental factors have varying degrees of influence, not just between taxa but also on an intraspecific spatial scale. For now, species specific studies, such as this thesis, provide our best insight into the kaleidoscopic complexity of evolutionary processes.

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Research impact & management recommendations

Despite being one of the most well-known marine animals in the world, Bottlenose Dolphins *Tursiops truncatus* have only been studied in a small percentage of the Mediterranean Sea (mostly in northern and coastal areas) (Bearzi *et al*., 2009). As such they remain fundamentally understudied and lack of data on genetic structure, movements and ecology limits conservation potential. The IUCN lists research on population size, distribution and trends as a priority for research (IUCN, 2009) and this study contributes towards this aim.

Both ACCOBAMS and the IUCN list *T. truncatus* as Vulnerable in the Mediterranean and Threatened within the Black Sea (ACCOBAMS Scientific Committee, 2007). However, in both organisations Mediterranean *T. truncatus* are treated as a single management unit. This study has shown that a) Mediterranean *T. truncatus* cannot be treated in isolation from Atlantic populations, b) within the Mediterranean there is complex population structure with localised populations and cannot be interpreted as a single unit and c) differential feeding ecology across this region for *T. truncatus* means that the potential impact of conservation measures is likely to be population specific.

Bearzi *et al*. (2009) notes that a variety of conservation measures are already embedded within existing legislation and international treaties but there is lack of compliance and enforcement. In light of the results of this study it is recommended that this existing legislation is reviewed for its capacity to treat *T. truncatus* within the Mediterranean as separate populations. There must then be a willingness for nations to work collaboratively (including the autonomous Macaronesian regions) and to enforce any conservation measures enacted.

Appendices

Appendix I - Desktop laboratory protocol – ddRADseq

Step 1: DNA Extraction & Quantification
Phenol-Choloroform DNA extraction
ALL EXTRACTION WORK TO BE CARRIED OUT IN A FUME CUPBOARD
Before starting you will need:
Buffer:
Tris-HCl 1M
NaCl 1M
EDTA 0.5 M
Combine these to make TNE buffer:
TNE buffer (10mM Trs-HCl / 0.1M NaCl / 1 mM EDTA)
For digestion (day 1):
SDS (10%)
Proteinase K^+ (20mg/ml)
TNE buffer (10mM Trs-HCl / 0.1M NaCl / 1 mM EDTA) – see above.

For extraction (day 2):
100% ethanol
Chloroform/Iso-Amylic Alcohol (24:1)
Sodium Acetate (3M)
70% cold ethanol
TE (10mM Tris-HCl / 1mM EDTA)

Day 1
Add approximately 0.1g of tissue to a fresh Eppendorf tube.
Add 500µl TNE
Add 100 µl Tris-HCl
Add 150 µl NaCl
Add 100 µl SDS
Add 10 µl Proteinase K^+
Gently vortex to mix well and then incubate for at least two hours in a water bath at 55°C (overnight is better).

Day 2
Make up a 24:1 mix of Chloroform and Iso-Amylic Alcohol. Iso-Amylic Alcohol is sometimes called 3-Methylbutanol.
Store your 70% ethanol on ice or in -20°C freezer.
Add 0.5x sample volume Phenol to your Eppendorf.
Add 0.5x volume Chloroform/Iso-Amylic Alcohol (24:1) to your Eppendorf.
Strongly mix for at least 10 minutes. This step is critical. It is best to continuously upend and right your tube by hand. Do not use a vortex.
Centrifuge at >12,000 rpm for 10 minutes.
Transfer the supernatant to fresh tubes using a pipette.

Add 1x volume Chloroform/Iso-Amylic Alcohol (24:1) to your new Eppendorf containing the supernatant. Note no phenol is added in this step.
Strongly mix for at least 10 minutes. This step is critical. It is best to continuously upend and right your tube by hand. Do not use a vortex.
Centrifuge at >12,000 rpm for 10 minutes.
Transfer the supernatant to fresh tubes using a pipette.
Add 2x volume ethanol (100%) to your Eppendorf.
Add 2% of 2x volume Sodium acetate (3M) to your Eppendorf. For example to a 50ul sample add 100ul 100% ethanol and 2ul 3M Sodium acetate.
Place samples in the freezer at -20 ^{o}C for 30 minutes. Store overnight for suspected low yield samples.
Centrifuge at 12,000 rpm for 10 minutes.
Remove the supernatant and discard.
Add 2x volume cold ethanol (70%)
Centrifuge at 12,000 for 5 minutes.
Remove the supernatant and discard.
Dry the pellet by placing <u>open</u> Eppendorfs in vacuum centrifuge. Ensure centrifuge is spinning before switching on the vacuum to prevent loss of samples.This step should be for as long as necessary to ensure that samples are completely dry. This step is crucial.
Add 50 µl TE buffer. Your extracted DNA should now be suspended in this.

Qubit
Estimate DNA concentration of samples using a Qubit 2.0 Fluorimeter
Make up your fluorescent master mix (See Figure S1.1) by the following equation: *(Number of samples + number of standards = n) x 199ul Qubit buffer + n x 1ul Qubit reagent*
Add 190ul Qubit mastermix to two standard Qubit tubes. Add 199ul Qubit mix to each sample Qubit tube. Add 10ul of each standard to the relevant tube. Add 1ul of each sample to the relevant tube. Mix gently and leave for two minutes. Take Qubit measurements immediately after two minutes reaction time is complete. Adjust Qubit fluorimeter to calculate for 1ul sample and to give measurement in ng/ul.

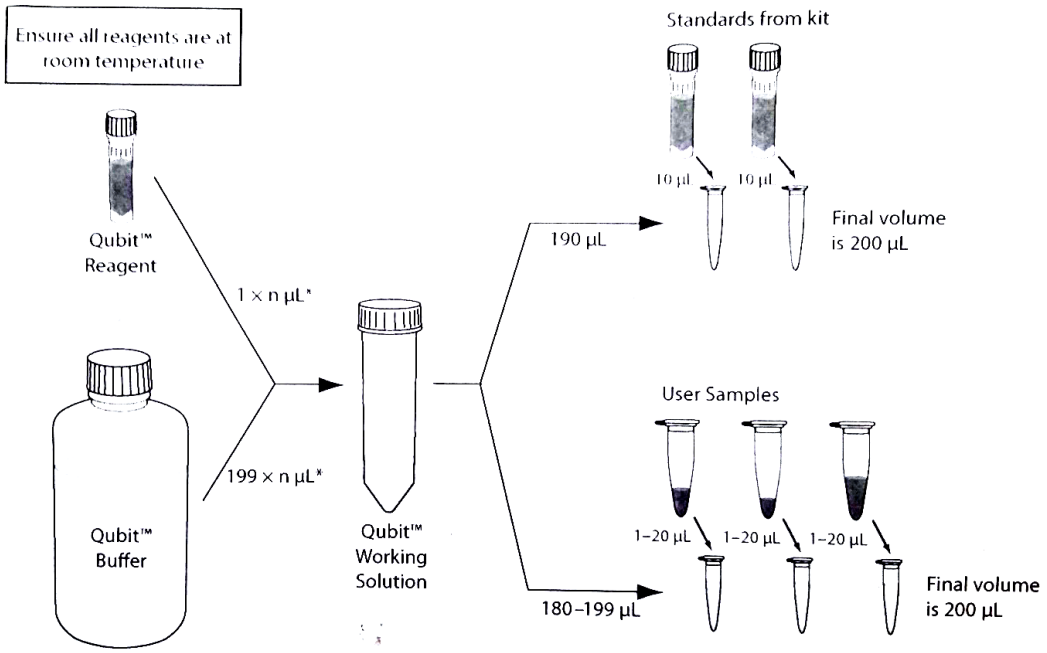


Figure S1.6: Preparation of samples and reagents for Qubit measurement. Figure designed by Thermo-Fisher Scientific.

This step may be required two or three times until you have consistent values. From Qubit values calculate volume that contains 250ng (or other required volume) DNA using the following equation:

Aliquot required for 250ng DNA = 250 / stock concentration (ng/µl)

Step 2: Library Prep
Restriction Digest
Restriction Digest can be done on all samples in one go in a PCR plate or in batches Draw a plate scheme of sample locations before commencing or ensure strip tubes are well labelled. If using a plate add 40µl PCR standard H_2O to each well. Then for each well set your pipette to the volume for 250ng DNA as calculated above. Remove this volume of water from the well and using the same tip (it has only been exposed to clean water) add your calculated quantity of DNA for that sample.

Make a master mix sufficient for all samples (n+1):

Reagent	µl per reaction
Buffer	5.0
BSA	0.5
Spermidine (50mM)	2.5
MspI (100,000 U/ml)	1.0
HindIII (100,000 U/ml)	1.0

Total reaction volume is 50µl.

Digest at 37°C overnight or for more rapid preparation 3hours will be sufficient for most DNA to have been digested.

Heatkill of enzymes is not necessary with an overnight digestion. If you choose to heatkill then run at 65°C for 20 minutes. Do not clean up – proceed directly to ligation.

After digest run 2-3ul of each sample on a gel. Visually inspect the gel image and adjust volumes of sample to be added to the ligation plate in order to ensure similar amounts of total DNA are added for each sample. For example: if sample A appears twice as bright as sample B on the gel then add half as much volume of digested sample A.

Ligation
Firstly prepare an excel sheet with the schematic of your ligation plate. Put your pools in rows. Ensure to make a note of which sample will get which barcode mix. If your pools are in rows then each column should receive the same barcode, thus enabling you to minimize risk of contamination by using strip caps.

Prepare your ligation mix (it is best to add a 10% error margin – i.e. for 60 samples make enough mastermix for 66):

Reagent	ul/tube
Buffer	4
T4 Ligase	0.5
H2O	10.5

Add 15ul of your ligation mix to each well of your ligation plate.
Taking your plate from digestion transfer the gel adjusted volumes of each sample to the relevant well in your ligation plate. add 5µl of unique adapter mix (P1 +P2 adaptor) mastermix to each sample within a designated group. i.e. for each pool you will use 12 different barcodes (if you have 12 samples in each pool, otherwise less).
Set up a thermocycler:

22°C	120mins
65°C	20mins
4°C	∞

After each ligation, pool remaining products.
At this stage you may want to *check the success of ligation by conducting an amplification* PCR using the illumine (p5 & p7) primers. Run 3ul of each completed reaction. You should see a large amount of DNA indicative of a successful ligation.

Qubit
Estimate ligated DNA concentration of samples using a Qubit 2.0 Fluorimeter
Make up your fluorescent master mix by the following equation:
(Number of samples + number of standards = n) x 199ul Qubit buffer + n x 1ul Qubit reagent
Add 190ul Qubit mastermix to two standard Qubit tubes. Add 199ul Qubit mix to each sample Qubit tube. Add 10ul of each standard to the relevant tube. Add 1ul of each sample to the relevant tube. Mix gently and leave for two minutes. Take Qubit measurements immediately after two minutes reaction time is complete. Adjust Qubit fluorimeter to calculate for 1ul sample and to give measurement in ng/ul.

Bead cleaning

Each bead solution should have been calibrated. Make sure you know what quantity you should add. A typical value is 1.8x volume.
Ensure the bead solution is homogenous by upending the tube several times until no beads remain in the bottom of the tube. This may need to be done before each aliquot.

Add 1.8x pool volume of bead solution to each pool. Mix in tubes by pipetting up and down several times. Incubate at room temperature for 5 minutes.
Place tubes on a magnet and remove supernatant. Retain this in a separate labelled tube.
Whilst on the magnet, add enough 80% ethanol to cover the bead pellet. Leave for 30 seconds before removing, again keep in a separate labelled tube. Repeat this step a second time.
Wait 5 to 20 minutes until beads are dry. Keep your samples on the magnet, with the lid of the tubes open. Before you continue to the next step, all ethanol should be evaporated. (Ethanol remains will interfere with following steps.) However, you also don't want to overdry your DNA (which tells by cracks in your pellets). So there is a balance
Take your sample off the magnet. Add 15 ul (or 10 ul) elution buffer in each sample, to release DNA from the beads. You might need to take the EB up and release it again several times to release the pellet from the wall. Mix by pipetting.
Place on magnet until beads have separated.
Carefully transfer the supernatant to a new tube. Try to obtain as much as possible to get the whole 15ul without disturbing the pellet. Repeat with a second 15ul EB. This supernatant should now contain your DNA suspended in 30ul.

Qubit
Estimate cleaned ligated DNA concentration of samples using a Qubit 2.0 Fluorimeter
Make up your fluorescent master mix by the following equation:
(Number of samples + number of standards = n) x 199ul Qubit buffer + n x 1ul Qubit reagent
Add 190ul Qubit mastermix to two standard Qubit tubes. Add 199ul Qubit mix to each sample Qubit tube. Add 10ul of each standard to the relevant tube. Add 1ul of each sample to the relevant tube. Mix gently and leave for two minutes. Take Qubit measurements immediately after two minutes reaction time is complete. Adjust Qubit fluorimeter to calculate for 1ul sample and to give measurement in ng/ul.

Pippin Prep
Ensure reagents are at room temperature.

Prepare samples ready for insertion.

Your sample should be in 15-40µl from the previous bead cleaning. If your DNA sample is below 40 µl then bring it up to 40 µl with TE. Take your 30µl of DNA sample and add to it 10µl of loading solution marked L. Vortex this sample and centrifuge. Retain the remaining 10ul of sample and keep it safe.

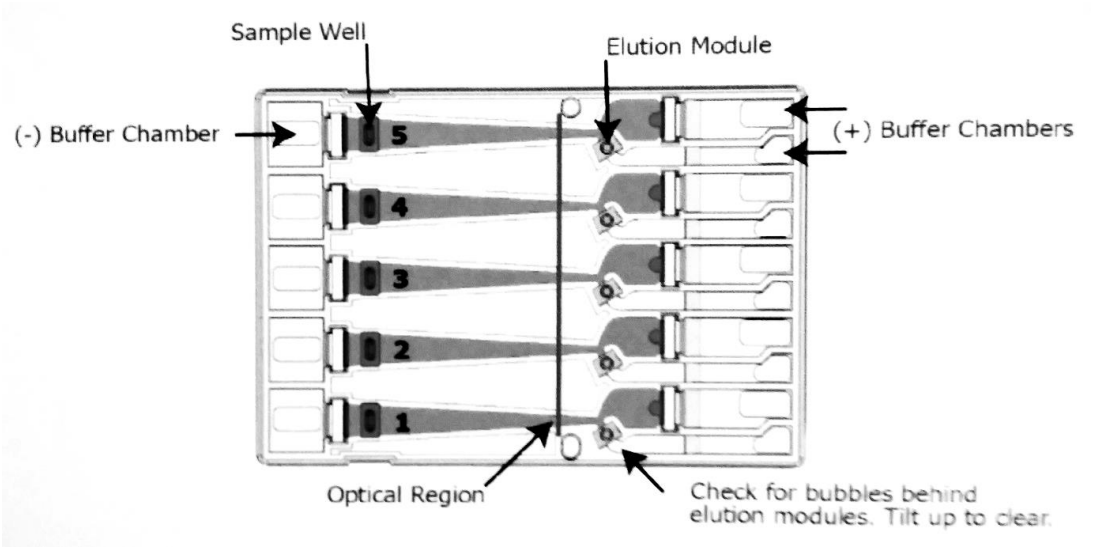


Figure S1.2: Layout of the Pippin Prep cartridge. Diagram designed by Sage Science

Setting up a program
Turn on the monitor and Pippin Prep machine. The Pippin has a small black button to the back right of the machine. Click the **Protocol Editor** Tab. Either load a previous protocol to edit **Load** or start from fresh **New**. Select Cassette type from the folder menu. This must be done first. Select '2% DF Marker L'. Adjust the time value if necessary. Elutions in the region of 300-500bp usually take about 1 – 1 ½ hours. Selecting 3 hours ensures the Pippin runs well past your selected bp window. Alternatively check the box for ending run after elution is complete.
Select the size selection protocol. There are 4 options:
Tight - collects minimum allowable distribution range of DNA fragments using the median target base pair value
Range - allows users to select the range to be collected using starting and ending base pair values.
Time - allows users to program extractions using the starting and ending elution time (hr:min:sec)
values only (a reference DNA marker is not used)

Peak - collects the next peak (restriction fragment or PCR band) after the set threshold base pair value has been reached.

We usually select either tight with the 'BP target' being your target length DNA or range and define your selection window. Assign the reference lane (select the same number as for each lane), ensuring any unused lanes are selected 'off'. We add 10µl of internal standard loading solution (L) to every 30µl sample so we don't need a reference lane. Instead we select **Use Internal Standards**. You must ensure this is done before clicking **Save As**.

Enter Sample ID or description. Click **Save As** and enter a name for this protocol before saving it in your personal folder.

Rinse the Pippin electrodes by filling the blank cassette with distilled water. Place in cradle and close lid. Hold for ten seconds before opening the lid, removing cassette

and disposing the water. Lightly remove any spilled water with paper towels, avoiding the optical nest.

Calibrate the Pippin by placing the calibration fixture onto the optical nest. Close the lid and press 'CALIBRATE' to open the calibration window. Enter 0.80 in the target window and click 'Calibrate'. When this is complete, click 'Exit'.

Remove the gel cassette (See Figure S1.2) from the foil packaging and inspect for gel columns. If there is a break in the gel do not use that lane. Inspect for bubbles and tap the cassette to clear them, making extra sure to clear bubbles around the elution wells. Place the cassette in the optical nest. Remove adhesive strips from cassette and top up buffer chambers with spare buffer.

Remove all buffer from elution wells and replace with ONLY 40ul fresh buffer. Seal these wells with fresh adhesive tape. Check the buffer wells in the sample wells and if necessary top up to full. Close the lid and perform the continuity test.

Re-check buffer level in the sample wells. Remove 40ul buffer from the sample wells and replace with your prepared sample. Close the lid and check the protocol again before pressing start.

After approx. 1hr20mins your run should be complete (for a 500bp selection). Remove your sample from the elution wells. Your sample should be in 40ul Tris-TAPS solution.

Qubit

Estimate cleaned size selected DNA concentration of samples using a Qubit 2.0 Fluorimeter

Make up your fluorescent master mix by the following equation:

(Number of samples + number of standards = n) x 199ul Qubit buffer + n x 1ul Qubit reagent

Add 190ul Qubit mastermix to two standard Qubit tubes. Add 199ul Qubit mix to each sample Qubit tube. Add 10ul of each standard to the relevant tube. Add 1ul of each sample to the relevant tube. Mix gently and leave for two minutes. Take Qubit measurements immediately after two minutes reaction time is complete. Adjust Qubit fluorimeter to calculate for 1ul sample and to give measurement in ng/ul.

Amplification

This step uses a unique index as the reverse primer which allows us later (after pooling) to identify which pool a given fragment came from. In combination with the earlier attached barcode allows us to identify an individual sample from which that fragment arrives. We set up a mastermix for each pool which is unique apart from the reverse primer (index).

This is best done in strip tubes. For each pool set up 4 20ul reactions, each containing 10ul of DNA from your Pippin extraction.

Each reaction will contain:

H2O	2.8ul
Phusion HF Buffer	4ul
dNTPs	0.44ul

10mM Forward Primer (PCR1)	1.28ul
10mM Reverse Primer (Index)	1.28ul
Phusion HF Polymerase	0.2ul
Size selected DNA	10ul

Set up and run the following thermal cycle on a PCR machine:

98°C	30sec
11 Cycles of:	
98°C	30sec
62°C	20sec
75°C	45sec
75°C	5min
4°C Hold	∞

Remove and pool your 4 reactions from each pool, ensuring each pool remains separate.

Qubit

Estimate cleaned amplified DNA concentration of samples using a Qubit 2.0 Fluorimeter

Make up your fluorescent master mix by the following equation:

(Number of samples + number of standards = n) x 199ul Qubit buffer + n x 1ul Qubit reagent

Add 190ul Qubit mastermix to two standard Qubit tubes. Add 199ul Qubit mix to each sample Qubit tube. Add 10ul of each standard to the relevant tube. Add 1ul of each sample to the relevant tube. Mix gently and leave for two minutes. Take Qubit measurements immediately after two minutes reaction time is complete. Adjust Qubit fluorimeter to calculate for 1ul sample and to give measurement in ng/ul.

Bead cleaning

NB. This last clean may be more suitable to column cleaning (expected 70% return) if your DNA concentration is high.

Each bead solution should have been calibrated. Make sure you know what quantity you should add. A typical value is 1.8x volume.

Ensure the bead solution is homogenous by upending the tube several times until no beads remain in the bottom of the tube. This may need to be done before each aliquot.

Add 1.8x pool volume of bead solution to each pool. Mix in tubes by pipetting up and down several times. Incubate at room temperature for 5 minutes.

Place tubes on a magnet and remove supernatant. Retain this in a separate labelled tube.

Whilst on the magnet, add enough 80% ethanol to cover the bead pellet. Leave for 30 seconds before removing, again keep in a separate labelled tube. Repeat this step a second time.

Wait 5 to 20 minutes until beads are dry. Keep your samples on the magnet, with the lid of the tubes open. Before you continue to the next step, all ethanol should be evaporated. (Ethanol remains will interfere with following steps.) However, you also don't want to over-dry your DNA (which tells by cracks in your pellets). Try to find the right balance.
Take your sample off the magnet. Add 15 ul (or 10 ul) elution buffer in each sample, to release DNA from the beads. You might need to take the EB up and release it again several times to release the pellet from the wall. Mix by pipetting.
Place on magnet until beads have separated.
Carefully transfer the supernatant to a new tube. Try to obtain as much as possible to get the whole 15ul without disturbing the pellet. Repeat with a second 15ul EB. This supernatant should now contain your DNA in 30ul EB.

Tapestation
Use D1000 reagents – D1000 Buffer (●) & D1000 Ladder (●)
Allow all reagents to equilibrate to room temperature for 30 minutes.
Remember to fill in Tapestation usage sheet found in Screen Tape box

Log on to the computer, username and password is found on the computer.
Launch Tapestation controller software
Load the Screen tape (found in the fridge) into the Tapestation
Load the loading tips (found in the Tapestation drawer) into the Tapestation. Fill all tip wells even if you have less samples.

Put strip tubes into tube rack
Add 3µl D1000 Buffer to each tube.
Add 1µl DNA **or** D1000 ladder to each tube
Add lids to tubes. Mark Tube 1 & 8 with pen.

There is no need to vortex the tubes as the volumes are so small, a simple flick will suffice.
Quickly spin the tubes so that all liquid rests in the bottom of the tube.

Place the tube strips into the Tapestation so that the ladder is in the top left well.
Remove the lids from the strip tubes. Close the Tapestation lid.

On the computer, select at least two wells for sampling. Check the top left well as the ladder. Add sample labels to all selected wells. Click **Start**.
Assign a file name and pathway.

This typically takes 10 minutes. Upon completion, use the computer to estimate molarity of your size selected peak.
qPCR

The qPCR is used to accurately estimate the concentration of your pools before final pooling. The results should be interpreted alongside your qubit results as a guide. Whenever possible try to work with others for the qPCR as standards are extremely expensive.

Firstly you need to set up your dilutions. If your Qubit results suggest your concentration is above 50ng/ul you may need to first create a 1 in 10 dilution of your pool to ensure your final dilutions fall in range of the qPCR standards.

You should make two independent replicates of a 1 in 1000 and 1 in 5000 dilution for each pool. Dilutions should be made up slowly, typically over about an hour. To do this take six new Eppendorfs for each pool. In the first two, labelled A & B add 99ul **Tris** buffer. Use Tris as EDTA (found in TE) can impede PCR. To the second, labelled C & D add 9ul Tris buffer. To the final two, labelled E & F, add 49ul Tris buffer. Add 1ul of your pooled DNA to both A & B. Mix by pipetting and leave for 15mins. Next add 1ul of A to both C & E. Add 1ul of B to D & F. Mix by pipetting and leave for 15 minutes. C & D now contain independent 1 in 1000 dilutions and E & F contain independent 1 in 5000 dilutions.

qPCR is conducted in a white well PCR plate.

Pools can now be pooled to equal molarity based on qPCR results.

Appendix II - Desktop lab protocol – Stable Isotope Analysis

Step 1: Lipid Extraction

You will need:

3:1 Dichloromethane:Methanol (4ml x n)
Eppendorfs (1 x n)
15ml tube (1 x n)
Gloves
Sharpie
De-ionized water

Method

- From the sample tube remove a tissue piece approximately 0.25cm^3. place this in a fresh and labelled Eppendorf.
- Finely dice the tissue with scissors.
- Add 1ml of 3:1 Dichoromethane:Methanol to the Eppendorf. Prepare a batch of samples.
- Sonicate for 15 minutes in a water bath.
- Centrifuge at 3000rpm for 10 minutes.
- Remove excess water.
- Repeat the above 4 steps twice more.
- The remaing solid sample should then be sonicated in 1ml deionized water for 15 minutes before being centrifuged at 3000rpm for ten minutes.
- Remove excess water.
- Air dry samples in a drying cupboard at 50°C for 48 hours.

Step 2: Weighing samples

You will need:

Fine tipped forceps x2
6x4mm tin capsules
Small pestle (metal)
Micro lab scoop
High sensitivity analytical balance

Method

- Ensure working surface and all instruments are thoroughly cleaned (acetone)
- Crush sample in to a fine powder, within the Eppendorf, using the small pestle
- Place an empty tin capsule on the balance and tare

- Using the fine forceps and scoop weigh out 0.3-0.4mg of sample in to the capsule
- Note final weight down.
- Remove the capsule containing sample from the balance and press in to a tight cube shape using the two pairs of forceps.
- Place prepared samples individually in to a 96 well plate ready for analysis.
- Ensure all instruments are cleaned between each sample.

Appendix III - Sample metadata

Tursiops truncatus

Use: 1 = Genetics only, 2 = Stable isotopes only & 3 = Both

Sample	Long.	Lat.	Location	Country	Sea	Source	Sex	Date	Tissue	Storage	Sampling	Use
AZ101	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	11/07/2006	Skin	Ethanol	Biopsy	3
AZ103	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	13/07/2006	Skin	Ethanol	Biopsy	3
AZ104	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	13/07/2006	Skin	Ethanol	Biopsy	3
AZ105	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	13/07/2006	Skin	Ethanol	Biopsy	3
AZ108	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	18/08/2007	Skin	Ethanol	Biopsy	1
AZ109	-28.7619	38.56228	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	23/02/2014	Muscle	Ethanol	Stranding	3
AZ2	-28.7619	38.56228	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	26/04/2002	Skin	Ethanol	Biopsy	3
AZ3	-28.5447	38.47889	Pico, Azores	Portugal	Atlantic	Mónica Silva	M	03/05/2002	Muscle	Ethanol	Biopsy	3
AZ40	-28.5982	38.57719	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	13/06/2003	Muscle	Ethanol	Biopsy	1
AZ41	-28.5982	38.57719	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	13/06/2003	Muscle	Ethanol	Biopsy	3
AZ44	-28.5982	38.57719	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	04/07/2003	Muscle	Ethanol	Biopsy	1
AZ45	-28.5982	38.57719	Faial, Azores	Portugal	Atlantic	Mónica Silva	M	04/07/2003	Muscle	Ethanol	Biopsy	3
AZ46	-28.5745	38.53961	Faial-Pico Channel	Portugal	Atlantic	Mónica Silva	M	22/07/2003	Muscle	Ethanol	Biopsy	1
AZ48	-28.5447	38.47889	Pico, Azores	Portugal	Atlantic	Mónica Silva	M	01/08/2003	Muscle	Ethanol	Biopsy	3
AZ54	-28.5447	38.47889	Pico, Azores	Portugal	Atlantic	Mónica Silva	M	15/08/2003	Muscle	Ethanol	Biopsy	3
AZ6	-28.5745	38.53961	Faial-Pico Channel	Portugal	Atlantic	Mónica Silva	F	04/05/2002	Muscle	Ethanol	Biopsy	1
AZ67	-28.5447	38.47889	Pico, Azores	Portugal	Atlantic	Mónica Silva	M	16/10/2003	Muscle	Ethanol	Biopsy	1
AZ8	-28.5447	38.47889	Pico, Azores	Portugal	Atlantic	Mónica Silva	M	01/07/2002	Muscle	Ethanol	Biopsy	3
AZ83	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	20/09/2005	Muscle	Ethanol	Biopsy	3
AZ84	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	20/09/2005	Muscle	Ethanol	Biopsy	3

AZ85	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	20/09/2005	Muscle	Ethanol	Biopsy	1
AZ86	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	20/09/2005	Muscle	Ethanol	Biopsy	1
AZ87	-31.6013	37.97134	Princess Alice Bank	Portugal	Atlantic	Mónica Silva	M	20/09/2005	Muscle	Ethanol	Biopsy	1
AZ88	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	06/07/2006	Muscle	Ethanol	Biopsy	1
AZ89	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	06/07/2006	Muscle	Ethanol	Biopsy	1
AZ92	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	06/07/2006	Muscle	Ethanol	Biopsy	1
AZ95	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	11/07/2006	Muscle	Ethanol	Biopsy	1
AZ97	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	11/07/2006	Muscle	Ethanol	Biopsy	3
AZ99	-31.1445	39.50358	Flores, Azores	Portugal	Atlantic	Mónica Silva	M	11/07/2006	Muscle	Ethanol	Biopsy	3
T19	-0.03074	38.59625	Altea	Spain	Balearic Sea	Alex Aguilar	M	22/04/1994	Skin	DMSO	Biopsy	1
19G	-6.85593	37.10835	Mazagon	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
20GP	-6.85593	37.10835	Mazagon	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
21GE	-6.85593	37.10835	Mazagon	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
25GP	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
30GE	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
34GP	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
36GPC	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
37GPC	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
42G	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
43GE	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
44G	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
45GE	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
46G	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
47GE	-6.41118	36.52347	Cádiz	Spain	Gulf of Cádiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
RB12	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2004	Unk.	Unk.	Unk.	1
RB14	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2004	Unk.	Unk.	Unk.	1
RB29	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2004	Unk.	Unk.	Unk.	1

RB30	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
RB32	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
RB33	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
RB34	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
RB35	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	3
RB37	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	Elsa	Unk.	2005	Unk.	Unk.	Unk.	1
RV3pE	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	CREMA	Unk.	2000	Unk.	Unk.	Unk.	3
RV7m	-5.55731	36.01252	Estrecho	Spain	Gibralter Strait	CREMA	Unk.	2000	Unk.	Unk.	Unk.	3
RB19	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB2	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB20	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB21	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB22	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB23	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB24E	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB25	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB26	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	3
RB39	-3.61051	36.67973	Granada	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB4	-2.44384	36.73353	Almería	Spain	Alborán Sea	Toftevaag	Unk.	2004	Unk.	Unk.	Unk.	1
RB45	-3.03579	35.93925	Alborán	Spain	Alborán Sea	Toftevaag	Unk.	2005	Unk.	Unk.	Unk.	1
T17	-0.63618	38.10114	Guardamar	Spain	Alborán Sea	Alex Aguilar	F	15/01/1997	Muscle	Unk.	Unk.	1
T20	0.419275	40.35708	Peñíscola	Spain	Balearic Sea	Alex Aguilar	M	25/03/2000	Skin	DMSO	Unk.	1
T28	2.806675	41.66221	Blanes	Spain	Balearic Sea	Alex Aguilar	M	16/12/1993	Skin	DMSO	Unk.	1
T29	2.806675	41.66221	Blanes	Spain	Balearic Sea	Alex Aguilar	F	04/05/1993	Skin	DMSO	Unk.	1
T3	0.07384	40.00882	Castellón	Spain	Balearic Sea	Alex Aguilar	F	12/06/1992	Skin	DMSO	Unk.	1
T43	1.298667	39.0728	Balleares	Spain	Balearic Sea	Alex Aguilar	F	01/01/2001	Unk.	Unk.	Biopsy	1
T7	-0.37432	38.423	Campello	Spain	Alborán Sea	Alex Aguilar	M	06/05/1993	Skin	DMSO	Unk.	1

T8	2.132179	41.28517	Mercabarna	Spain	Balearic Sea	Alex Aguilar	F	16/12/1988	Skin	DMSO	Unk.	1
T9	3.154816	42.19014	Empúries	Spain	Balearic Sea	Alex Aguilar	F	09/04/1989	Skin	DMSO	Unk.	1
VAL1	-0.5048	38.17243	Santa Pola	Spain	Balearic Sea	Toni Raga	M	04/03/2009	Skin	Frozen	Stranding	3
VAL10	-0.63618	38.10114	Guardamar	Spain	Balearic Sea	Toni Raga	F	12/06/2014	Skin	Frozen	Stranding	3
VAL2	-0.49878	38.2394	Elche	Spain	Balearic Sea	Toni Raga	M	23/03/2011	Skin	Frozen	Stranding	3
VAL3	-0.33322	39.41818	Pinedo	Spain	Balearic Sea	Toni Raga	F	14/07/2010	Skin	Frozen	Stranding	3
VAL4	-0.0407	38.60353	Altea	Spain	Balearic Sea	Toni Raga	M	11/07/2008	Skin	Frozen	Stranding	3
VAL5	-0.70174	37.95775	Torrevieja	Spain	Balearic Sea	Toni Raga	M	25/06/2010	Skin	Frozen	Stranding	1
VAL6	-0.19996	39.66816	Sagunto	Spain	Balearic Sea	Toni Raga	F	19/03/2011	Skin	Frozen	Stranding	3
VAL7	-0.64498	38.07087	Orihuela	Spain	Balearic Sea	Toni Raga	F	18/08/2017	Skin	Frozen	Stranding	3
CL17	11.18337	42.46276	Orbetello	Italy	Thyrrenian	Marsili Letizia	F	17/11/1996	Skin	Frozen	Stranding	1
CL551	10.50248	43.28946	Tuscany	Italy	Thyrrenian	Marsili Letizia	M	26/06/1990	Skin	Frozen	Stranding	1
CL59	10.29758	43.53617	Livorno	Italy	Thyrrenian	Marsili Letizia	Unk.	24/05/1990	Heart	Frozen	Stranding	1
GB183	12.56938	37.65721	Mazara Del Vello	Italy	Mediterranean	Giusi Buscaino	F	10/05/2000	Skin	DMSO	Stranding	1
SIC01	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	06/09/2017	Skin	DMSO	Biopsy	3
SIC02	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	06/09/2017	Skin	DMSO	Biopsy	3
SIC03	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	07/09/2017	Skin	DMSO	Biopsy	3
SIC05	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	07/09/2017	Skin	DMSO	Biopsy	3
SIC06	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	07/09/2017	Skin	DMSO	Biopsy	3
SIC09	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	10/09/2017	Skin	DMSO	Biopsy	3
SIC12	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	12/09/2017	Skin	DMSO	Biopsy	3
SIC13	12.64775	37.56785	Torretta Granitola	Italy	Mediterranean	Daniel Moore	Unk.	18/09/2017	Skin	DMSO	Biopsy	3
TUS28	10.50248	43.28946	Tuscany	Italy	Thyrrenian	Ada Natoli	M	27/06/1994	Skin	Frozen	Stranding	1
TtLAZ1	12.22375	41.76242	Rome	Italy	Thyrrenian	MMTB	M	30/06/2011	Muscle	DMSO	Stranding	1
TtSIC3	12.5794	37.6401	Mazara Del Vello	Italy	Mediterranean	Giusi Buscaino	F	10/06/2004	Skin	DMSO	Stranding	1
TtSIC4	12.58529	35.52302	Lampedusa	Italy	Mediterranean	Uni. of Siena	M	01/07/2006	Skin	DMSO	Stranding	1
TtTUS1	10.1851	43.8582	Viareggio	Italy	Thyrrenian	Uni. of Siena	Unk.	13/03/2008	Skin	DMSO	Stranding	1

TtTUS10	10.2133	43.5439	Livorno	Italy	Thyrrenian	MMTB	M	17/12/2009	Muscle	DMSO	Stranding	1
TtTUS14	10.2299	42.8391	Elba	Italy	Thyrrenian	Uni. of Siena	M	08/06/1999	Muscle	Lyophillised	Stranding	3
TtTUS2	10.1851	43.8582	Viareggio	Italy	Thyrrenian	Uni. of Siena	Unk.	14/03/2008	Skin	DMSO	Stranding	1
TtTUS20	10.21942	43.54789	Meloria	Italy	Thyrrenian	Uni. of Siena	F	24/05/1990	Muscle	Lyophillised	Stranding	3
TtTUS3	10.2133	43.5439	Livorno	Italy	Thyrrenian	Uni. of Siena	Unk.	17/12/2009	Skin	DMSO	Unk.	1
TtTUS38	11.1414	42.4554	Orbetello	Italy	Thyrrenian	Ada Natoli	F	18/11/2000	Unk.	Frozen	Stranding	1
TtTUS4	11.09336	42.43067	Monte Argentario	Italy	Thyrrenian	Banca Tessuti	M	17/05/2007	Muscle	DMSO	Unk.	1
TtTUS7	10.10941	43.99943	Antignano	Italy	Thyrrenian	Banca Tessuti	F	13/03/2008	Muscle	DMSO	Unk.	1
TtTUS8	10.10941	43.99943	Antignano	Italy	Thyrrenian	Banca Tessuti	F	14/03/2008	Muscle	DMSO	Unk.	1
TtTUS9	10.48984	43.29798	Marina di Cecina	Italy	Thyrrenian	Banca Tessuti	M	07/10/2008	Muscle	DMSO	Unk.	1
2_97	20.8702	38.6201	Kalamos	Greece	Aegean	Stefania Gaspari	M	29/08/1996	Teeth	Unk.	Stranding	1
2_99	20.8702	38.6201	Kalamos	Greece	Aegean	Ada Natoli/TRI	F	21/06/1999	Skin	DMSO	Biopsy	1
3_97	20.8702	38.6201	Kalamos	Greece	Aegean	Ada Natoli/TRI	M	29/08/1997	Teeth	Unk.	Stranding	1
6_97	20.8702	38.6201	Kalamos	Greece	Aegean	Ada Natoli/TRI	F	11/10/2001	Skin	DMSO	Biopsy	1
CL529	12.30326	45.21086	Chioggia	Italy	Adriatic	Marsili Letizia	Unk.	16/08/1992	Skin	Frozen	Unk.	1
CL540	12.94528	43.89593	Pesaro	Italy	Adriatic	Marsili Letizia	M	16/08/1992	Skin	Frozen	Stranding	1
CL541	12.3676	44.24975	Forli	Italy	Adriatic	Marsili Letizia	F	20/08/1992	Skin	Frozen	Stranding	3
CL542	12.39737	44.20935	Cesanatico	Italy	Adriatic	Marsili Letizia	F	11/04/1992	Skin	Frozen	Stranding	1
EPLIDO	12.37673	45.40981	Lido Venezia	Italy	Adriatic	Tethys	F	2000	Skin	DMSO	Unk.	1
FILIPPO	15.90232	41.60957	Manfredonia	Italy	Adriatic	Tethys	M	30/10/1998	Skin	DMSO	Unk.	1
G20	14.64953	44.69651	Unk.	Croatia	Adriatic	Tethys	F	12/10/2001	Muscle	Lyophillised	Stranding	1
INCOGNI.	14.64953	44.69651	Unk.	Croatia	Adriatic	Ada Natoli/TRI	Unk.	Unk.	Skin	DMSO	Stranding	1
T13	0.590146	40.60643	San Carles	Spain	Balearic	Alex Aguilar	F	1994	Skin	DMSO	Unk.	1
T16	-0.57232	38.18723	Santa Pola, Alicante	Spain	Alborán	Alex Aguilar	M	1992	Skin	DMSO	Unk.	1
T21	-0.2287	38.50012	Villajoyosa	Spain	Alborán	Alex Aguilar	F	1992	Skin	DMSO	Unk.	1
TRI006	14.39224	44.69649	Osor, Island of Cres	Croatia	Adriatic	Tethys	M	20/10/1994	Skin	DMSO	Stranding	1
TRI011	14.24478	44.6357	Unije Island	Croatia	Adriatic	Tethys	F	31/07/1997	Skin	DMSO	Stranding	1

TRI014	14.64953	44.69651	Unk.	Croatia	Adriatic	Tethys	F	13/07/1999	Skin	DMSO	Stranding	1
TUR1	20.8702	38.6201	Kalamos	Greece	Aegean	Tethys	M	22/08/1993	Skin	DMSO	Stranding	1
TURC1	20.8702	38.6201	Kalamos	Greece	Aegean	Tethys	F	09/10/1993	Skin	DMSO	Biopsy	1
TtGRE1	20.6054	38.9324	Mytikas	Greece	Ionian	Banca Tessuti	F	03/09/2007	Muscle	DMSO	Stranding	3
TtGRE14	20.87535	37.72433	Zakynthos	Greece	Ionian	Alexadrox Fazis	M	22/07/2009	Skin	DMSO	Unk.	1
TtGRE18	20.8702	38.6201	Kalamos	Greece	Aegean	Ada Natoli	M	22/08/1993	Skin	DMSO	Unk.	1
TtGRE20	20.8702	38.6201	Kalamos	Greece	Aegean	Ada Natoli	M	22/08/1994	Skin	DMSO	Stranding	1
TtMAR1	12.7901	43.9341	Pesaro	Italy	Adriatic	Marsili Letizia	M	16/08/1992	Skin	Frozen	Unk.	1
TtPUG4	16.1861	41.5865	Manfredonia	Italy	Adriatic	Tethys	M	30/10/1998	Skin	Frozen	Unk.	1
TtROM18	12.3201	44.3444	Cervia	Italy	Adriatic	MMTB	M	17/05/2011	Muscle	DMSO	Unk.	1
TtROM19	12.5088	44.1226	Rimini	Italy	Adriatic	MMTB	M	06/07/2011	Muscle	DMSO	Unk.	1
TtROM7	12.3233	44.2922	Forli	Italy	Adriatic	Marsili Letizia	F	20/08/1992	Skin	Frozen	Unk.	1
TtROM9	12.3273	44.2921	Cesanatico	Italy	Adriatic	Ada Natoli	F	12/04/1996	Skin	Frozen	Stranding	1
TtSLO1	13.5542	45.5398	Unk.	Slovenia	Adriatic	Tilen Genov	M	03/09/2011	Skin	Ethanol	Biopsy	1
TtSLO3	13.5542	45.5398	Unk.	Slovenia	Adriatic	Tilen Genov	M	07/09/2011	Skin	Ethanol	Biopsy	1
TtSLO5	13.5542	45.5398	Unk.	Slovenia	Adriatic	Tilen Genov	M	07/09/2011	Skin	Ethanol	Biopsy	1
TtSLO7	13.5542	45.5398	Unk.	Slovenia	Adriatic	Tilen Genov	F	07/09/2011	Skin	Ethanol	Biopsy	1
TtVEN1	12.48472	45.35694	Chioggia	Italy	Adriatic	Marsili Letizia	M	16/08/1992	Skin	Frozen	Unk.	1
TtVEN13	12.35111	45.10274	Rosolina	Italy	Adriatic	MMTB	F	09/09/2010	Muscle	DMSO	Unk.	1
Tur2	20.91822	38.60966	Kalamos	Greece	Ionian Sea	Tethys	M	22/08/1994	Skin	DMSO	Stranding	1
BS10	35.24917	44.91632	Kara Dag Reserve	Russia	Black Sea	Alexei Birkun	F	24/03/2002	Skin	DMSO	Unk.	1
BS11	35.24917	44.91632	Kara Dag Reserve	Russia	Black Sea	Alexei Birkun	M	24/03/2002	Skin	DMSO	Unk.	1
BS2	34.35758	44.57537	Partenit	Russia	Black sea	Alexei Birkun	M	12/05/2002	Skin	DMSO	Unk.	1
BS3	34.17402	44.48895	Yalta	Russia	Black Sea	Alexei Birkun	M	12/05/2002	Skin	DMSO	Unk.	1
BS4	34.17402	44.48895	Yalta	Russia	Black Sea	Alexei Birkun	F	12/05/2002	Skin	DMSO	Unk.	1
BS5	33.37168	45.17442	Evpatoria	Russia	Black Sea	Alexei Birkun	M	17/03/2002	Skin	DMSO	Unk.	1
BS6	33.37168	45.17442	Evpatoria	Russia	Black Sea	Alexei Birkun	M	17/03/2002	Skin	DMSO	Unk.	1

BS7	33.37168	45.17442	Evpatoria	Russia	Black Sea	Alexei Birkun	M	17/03/2002	Skin	DMSO	Unk.	1
BS8	33.37168	45.17442	Evpatoria	Russia	Black Sea	Alexei Birkun	F	17/03/2002	Skin	DMSO	Unk.	1
BS1	34.35758	44.57537	Partenit	Russia	Black sea	Alexei Birkun	M	12/05/2002	Skin	DMSO	Unk.	1
CRO101	14.4795	44.6057	Punta Kriza	Croatia	Adriatic	BWI - Drasko	F	24/08/2010	Skin	DMSO	Unk.	2
CRO24	14.0436	44.8203	Premantura	Croatia	Adriatic	BWI - Drasko	F	21/01/2000	Skin	DMSO	Unk.	2
CRO35	14.2443	44.6355	Sesnja	Croatia	Adriatic	Ada Natoli	Unk.	29/08/2001	Skin	DMSO	Stranding	2
CRO45	15.2501	44.1002	Zabodarski	Croatia	Adriatic	BWI - Drasko	Unk.	14/07/2003	Skin	Ethanol	Unk.	2
CRO57	14.8512	44.3544	Silba	Croatia	Adriatic	BWI - Drasko	Unk.	17/08/2006	Skin	DMSO	Unk.	2
CRO74	14.6254	43.6801	Silba	Croatia	Adriatic	BWI - Drasko	M	06/11/2007	Skin	DMSO	Unk.	2
MAR1	12.8777	43.7601	S. Bartolo	Italy	Adriatic	Ada Natoli	M	05/07/1996	Muscle	Frozen	Stranding	2
CRO18	14.4795	44.6057	Unk.	Croatia	Adriatic	Ada Natoli	F	31/07/1997	Muscle	Lyophillised	Stranding	2
AZ107	-28.5776	38.5348	Unk.	Portugal	Atlantic	Mónica Silva	Unk.	Unk.	Skin	Ethanol	Biopsy	2
AZ7	-28.5776	38.5348	Unk.	Portugal	Atlantic	Mónica Silva	Unk.	Unk.	Muscle	Ethanol	Biopsy	2
AZ90	-28.5776	38.5348	Unk.	Portugal	Atlantic	Mónica Silva	Unk.	Unk.	Muscle	Ethanol	Biopsy	2
AZ91	-28.5776	38.5348	Unk.	Portugal	Atlantic	Mónica Silva	Unk.	Unk.	Muscle	Ethanol	Biopsy	2
AZ93	-28.5776	38.5348	Unk.	Portugal	Atlantic	Mónica Silva	Unk.	Unk.	Muscle	Ethanol	Biopsy	2
GRE15	23.7647	38.8019	L.V. Attikis	Greece	Aegean	Alexadrox Fazis	M	17/10/2009	Skin	DMSO	Unk.	2
GRE10	23.375	38.975	Iraklion	Greece	Aegean	Alexadrox Fazis	Unk.	10/07/2006	Muscle	DMSO	Unk.	2
TUS5	10.2051	42.8186	Marciana	Italy	Thyrrenian	Banca Tessuti	F	03/10/2007	Muscle	DMSO	Unk.	2
VAL9	-0.32241	39.47416	Guardamar del Segura	Spain	Balearic	Toni Raga	M	11/07/2008	Muscle	Frozen	Stranding	2
CL546	12.3063	44.4598	Ravenna	Italy	Thyrrenian	Ada Natoli	M	24/08/1996	Skin	Frozen	Stranding	2
31G	-6.52499	36.9119	Unk.	Spain	S of Gulf of Cadiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	2
48G	-6.52499	36.9119	Unk.	Spain	S of Gulf of Cadiz	Elsa	Unk.	2005	Unk.	Unk.	Unk.	2
TUS13	10.9089	42.6983	Marina di Grosseto	Italy	Thyrrenian	Ada Natoli/Siena	F	24/03/2002	Unk.	Lyophillised	Unk.	2
TUS16	10.2133	43.5439	Livorno	Italy	Thyrrenian	Ada Natoli/Siena	F	26/07/1991	Kidney	Lyophillised	Unk.	2
TUS17	10.2133	43.5439	Livorno	Italy	Thyrrenian	Ada Natoli/Siena	F	05/05/1990	Liver	Lyophillised	Unk.	2
TUS27	10.2133	43.5439	Livorno	Italy	Thyrrenian	Ada Natoli/Siena	F	25/05/1994	Heart	Lyophillised	Stranding	2

TUS29	10.2151	43.8981	Lido di Camaiore	Italy	Thyrrenian	Ada Natoli/Siena	F	11/04/1994	Liver	Lyophillised	Stranding	2
TUS30	10.9089	42.6983	Marina di Grosseto	Italy	Thyrrenian	Ada Natoli/Siena	M	19/05/1995	Liver	Lyophillised	Stranding	2
TUS31	10.2133	43.5439	Livorno	Italy	Thyrrenian	Ada Natoli/Siena	M	27/07/1995	Liver	Lyophillised	Stranding	2
TUS5	10.2051	42.8186	Marciana Marina	Italy	Thyrrenian	Ada Natoli/Siena	F	03/10/2007	Muscle	DMSO	Unk.	2

Sphyraena viridensis

Use: 1 = Genetics only, 2 = Stable isotopes only & 3 = Both

Sample	**Long.**	**Lat.**	**Location**	**Country**	**Sea**	**Source**	**Date**	**Tissue**	**Storage**	**Sampling**	**Use**
Sv01	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv02	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv03	11.12939	42.44937	Argentario	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv04	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv05	10.33757	42.83182	Elba Island	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv06	11.12939	42.44937	Argentario	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv07	11.12939	42.44937	Argentario	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv08	17.91616	40.03739	Gallipoli	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv09	17.91616	40.03739	Gallipoli	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv10	17.91616	40.03739	Gallipoli	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv11	17.91616	40.03739	Gallipoli	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv12	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv13	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv14	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv15	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3

Sv16	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv17	15.31717	37.05688	Siracusa	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv18	9.26147	38.89958	Cagliari	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv19	9.26147	38.89958	Cagliari	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv20	9.26147	38.89958	Cagliari	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv21	9.26147	38.89958	Oristano	Italy	Sardinia	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv22	9.26147	38.89958	Cagliari	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv23	9.26147	38.89958	Cagliari	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv24	10.33757	42.83182	Elba Island	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv25	10.33757	42.83182	Elba Island	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv26	10.33757	42.83182	Elba Island	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv27	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv28	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv29	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv30	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv31	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv32	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv33	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv34	18.53393	40.14318	Otranto	Italy	Ionian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv35	11.86798	41.99828	Santa Marinella	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv36	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv37	11.12939	42.44937	Argentario	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	2
Sv38	11.12939	42.44937	Argentario	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv39	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv40	11.78412	42.08708	Civitavecchia	Italy	Tyrrhenian	Valentina Milana / Uni Roma	Unk.	Fin	ETOH	Unk.	3
Sv41	12.96678	40.90456	Ponza Island	Italy	Tyrrhenian	Armando Macali	Unk.	Muscle	ETOH	Pole & Line	2
Sv42	12.96678	40.90456	Ponza Island	Italy	Tyrrhenian	Armando Macali	Unk.	Muscle	ETOH	Pole & Line	3

Sv43	12.96678	40.90456	Ponza Island	Italy	Tyrrhenian	Armando Macali	Unk.	Muscle	ETOH	Pole & Line	3
Sv44	12.96678	40.90456	Ponza Island	Italy	Tyrrhenian	Armando Macali	Unk.	Muscle	ETOH	Pole & Line	2
Sv45a	13.23234	38.20500	Isola delle femmine	Italy	Tyrrhenian	Papasergi Salvatore	Unk.	Muscle	ETOH	Pole & Line	2
Sv45b	13.23234	38.20500	Isola delle femmine	Italy	Tyrrhenian	Papasergi Salvatore	Unk.	Muscle	ETOH	Pole & Line	-
Sv45c	13.23234	38.20500	Isola delle femmine	Italy	Tyrrhenian	Papasergi Salvatore	Unk.	Muscle	ETOH	Pole & Line	-
Sv46	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv46b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv47	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv47b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv48	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv48b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv49	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv49b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv50	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv50b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv51	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Muscle	ETOH	Market	3
Sv51b	2.66143	39.52417	Palma, Mallorca	Spain	Balearic Sea	Joan Moranta	Unk.	Fin	ETOH	Market	1
Sv52	5.34759	43.16812	Marseille	France	Balearic Sea	Mireille Harmelin-Vivien	07/12/2010	Muscle	Freeze-Dried	Spear Fishing	2
Sv53	5.34759	43.16812	Marseille	France	Balearic Sea	Mireille Harmelin-Vivien	07/12/2010	Muscle	Freeze-Dried	Spear Fishing	3
Sv54	5.34759	43.16812	Marseille	France	Balearic Sea	Mireille Harmelin-Vivien	07/12/2010	Muscle	Freeze-Dried	Spear Fishing	2
Sv55	5.34759	43.16812	Marseille	France	Balearic Sea	Mireille Harmelin-Vivien	07/12/2010	Muscle	Freeze-Dried	Spear Fishing	2
Sv56	5.34759	43.16812	Marseille	France	Balearic Sea	Mireille Harmelin-Vivien	07/12/2010	Muscle	Freeze-Dried	Spear Fishing	2
Sv57	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/Rui Elias	21/07/2017	Muscle	ETOH	Spear Fishing	3
Sv58	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/Rui Elias	21/07/2017	Muscle	ETOH	Spear Fishing	3
Sv59	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	28/07/2017	Muscle	ETOH	Spear Fishing	3
Sv60	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	28/07/2017	Muscle	ETOH	Spear Fishing	3
Sv61	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	28/07/2017	Muscle	ETOH	Spear Fishing	2

Sv62	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	02/09/2017	Muscle	ETOH	Spear Fishing	3
Sv63	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	02/09/2017	Muscle	ETOH	Spear Fishing	3
Sv64	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/João Medeiros	21/09/2017	Fin	ETOH	Spear Fishing	3
Sv65	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/João Medeiros	21/09/2017	Fin	ETOH	Spear Fishing	2
Sv66	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/João Medeiros	21/09/2017	Fin	ETOH	Spear Fishing	3
Sv67	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	21/09/2017	Fin	ETOH	Spear Fishing	2
Sv68	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	21/09/2017	Fin	ETOH	Spear Fishing	3
Sv69	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	21/09/2017	Fin	ETOH	Spear Fishing	2
Sv70	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/João Medeiros	21/09/2017	Fin	ETOH	Spear Fishing	2
Sv71	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/João Medeiros	21/09/2017	Fin	ETOH	Spear Fishing	3
Sv72	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/Tiago Silva	21/09/2017	Fin	ETOH	Spear Fishing	2
Sv73	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros/Tiago Silva	21/09/2017	Fin	ETOH	Spear Fishing	3
Sv74	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Fin	ETOH	Fishing Vesel	2
Sv75	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv76	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv77	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	2
Sv78	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv79	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv80	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv81	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv82	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	2
Sv83	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	2
Sv84	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv85	-28.0138	38.59111	Azores/SJZ	Portugal	Atlantic	João P. Barreiros	14/09/2017	Muscle	ETOH	Fishing Vesel	3
Sv86a	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Muscle	DMSO	Spear Fishing	3
Sv86b	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Muscle	DMSO	Spear Fishing	1
Sv86c	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Fin	DMSO	Spear Fishing	1

Sv87a	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Muscle	DMSO	Spear Fishing	3
Sv87b	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Muscle	DMSO	Spear Fishing	1
Sv87c	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	24/09/2017	Fin	DMSO	Spear Fishing	1
Sv88	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	26/09/2017	Fin	DMSO	Supermarket	2
Sv89	-27.1095	38.61127	Azores/TER	Portugal	Atlantic	João P. Barreiros	26/09/2017	Fin	DMSO	Supermarket	2
Sv90a	-27.1172	38.64693	Azores/TER	Portugal	Atlantic	João P. Barreiros	29/09/2017	Muscle	DMSO	Spear Fishing	2
Sv90b	-27.1172	38.64693	Azores/TER	Portugal	Atlantic	João P. Barreiros	29/09/2017	Muscle	DMSO	Spear Fishing	-
Sv90c	-27.1172	38.64693	Azores/TER	Portugal	Atlantic	João P. Barreiros	29/09/2017	Fin	DMSO	Spear Fishing	-
Sv91	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle/Skin	ETOH	Unk.	2
Sv92	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv93	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv94	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv95	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv96	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv97	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	2
Sv98	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	2
Sv99	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv100	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	2
Sv101	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv102	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv103	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3
Sv104	-16.2158	28.44937	Tenerife	Spain	Atlantic	Alberto Brito	Unk.	Muscle	ETOH	Unk.	3

Appendix IV – Bait-capture approach to develop an NGS microsatellite library

Appendix IV.I – Methodology: Library preparation, sequencing & bioinformatics

Production of a microsatellite capture library was completed using two sequential protocols. The first protocol, the so-called 'BEST protocol 2.0' was developed by Christian Carøe (pers. comm., 2018) and is based upon Carøe *et al*. (2018). The basis of this protocol is outlined for the reader below. This library was produced with 40 Italian samples, evenly split in origin either side of the Siculo-Tunisian Front to allow high resolution investigation in to the influence of this environmental barrier.

A volume of 32µl of extracted DNA was readied on ice, where 32µl of extracted DNA was not available then the extraction was diluted to this volume using elution buffer. The 32µl of extracted DNA was then added to 8µl of end-repair master mix in 0.2mL PCR strip-tubes. The end-repair master mix was prepared by combining 0.4µl T4 DNA polymerase (3U/µl), 1µl T4 PNK (10U/µl), 0.4µl dNTP (25mM), 4µl T4 DNA ligase buffer (10X, New England Biosystems) and finally 2.2µl reaction booster (prepared by adding 0.25g PEG-4000 to 100µl BSA (20mg/mL) and 80µl NaCl (5M) and made up to 1mL with deionized H_20). This mix, total reaction size 40µl, was then incubated for 30 minutes at 20°C using an Applied Biosystems Veriti 96 well thermal cycler before 2µl of appropriate adapter solution (see Table AV.1 for adapter details) was added to each tube. This was then followed by 30 minutes at 65°C before being cooled to 4°C. Once cooled, the reaction was mixed thoroughly by pipetting.

Table AV.1: Samples, adapters and indexes used to create the microsatellite capture library for *Sphyraena viridensis.*

Sample name	Adapter	Adapter seq.	Index	Index seq.
Sv1	P501	TCTAAGATCGG	P701	TAAGGCGA
Sv3	P502	AGAGAGATCGG	P701	TAAGGCGA
Sv4	P503	GATAAGATCGG	P701	TAAGGCGA
Sv5	P504	CTCTAGATCGG	P701	TAAGGCGA
Sv6	P505	TTACAGATCGG	P701	TAAGGCGA
Sv7	P506	CAGTAGATCGG	P701	TAAGGCGA
Sv8	P507	CCTTAGATCGG	P701	TAAGGCGA
Sv9	P508	TTAGAGATCGG	P701	TAAGGCGA
Sv10	P501	TCTAAGATCGG	P702	CGTACTAG
Sv11	P502	AGAGAGATCGG	P702	CGTACTAG
Sv12	P503	GATAAGATCGG	P702	CGTACTAG
Sv13	P504	CTCTAGATCGG	P702	CGTACTAG
Sv15	P505	TTACAGATCGG	P702	CGTACTAG
Sv17	P506	CAGTAGATCGG	P702	CGTACTAG
Sv21	P507	CCTTAGATCGG	P702	CGTACTAG
Sv22	P508	TTAGAGATCGG	P702	CGTACTAG
Sv23	P501	TCTAAGATCGG	P703	AGGCAGAA
Sv25	P502	AGAGAGATCGG	P703	AGGCAGAA
Sv26	P503	GATAAGATCGG	P703	AGGCAGAA
Sv27	P504	CTCTAGATCGG	P703	AGGCAGAA
Sv28	P505	TTACAGATCGG	P703	AGGCAGAA
Sv30	P506	CAGTAGATCGG	P703	AGGCAGAA
Sv31	P507	CCTTAGATCGG	P703	AGGCAGAA
Sv32	P508	TTAGAGATCGG	P703	AGGCAGAA
Sv33	P501	TCTAAGATCGG	P704	TCCTGAGC
Sv34	P502	AGAGAGATCGG	P704	TCCTGAGC
Sv35	P503	GATAAGATCGG	P704	TCCTGAGC
SV36	P504	CTCTAGATCGG	P704	TCCTGAGC
Sv39	P505	TTACAGATCGG	P704	TCCTGAGC
Sv40	P506	CAGTAGATCGG	P704	TCCTGAGC
Sv41	P507	CCTTAGATCGG	P704	TCCTGAGC
Sv42	P508	TTAGAGATCGG	P704	TCCTGAGC
Sv43	P501	TCTAAGATCGG	P705	GGACTCCT
Sv44	P502	AGAGAGATCGG	P705	GGACTCCT
Sv48	P503	GATAAGATCGG	P705	GGACTCCT
Sv50	P504	CTCTAGATCGG	P705	GGACTCCT
Sv51	P505	TTACAGATCGG	P705	GGACTCCT
Sv53	P506	CAGTAGATCGG	P705	GGACTCCT
Sv54	P507	CCTTAGATCGG	P705	GGACTCCT
Sv56	P508	TTAGAGATCGG	P705	GGACTCCT

Each mixed and cooled reaction was then made up to a total reaction volume of 50µl with 8µl of ligation master mix (made with 1µl T4 DNA ligase buffer (10X, New England Biosystems), 6µl PEG-4000 (50% solution) and 1µl T4 DNA ligase (400U/µl, New England Biosystems)) and incubated for 30 minutes at 20°C followed by 10 minutes at 65°C, then cooled to 4°C.

In a final reaction step 10µl of fill-in master mix was then added to give a new total volume of 10µl. The fill-in master mix was created by combining 2µl isothermal amplification buffer (10X), 0.8µl dNTP (25mM), 1.6µl of Bst 2.0 warmstart polymerase (8U/µl) and finally 5.6µl of molecular grade water. This was then incubated for 15 minutes at 65°C followed by 15 minutes at 80°C before being cooled to 4°C.

This library was then cleaned using SpeedBeads^{TM} carboxylate-modified magnetic particles (Sigma-Aldrich). Following bead calibration, 2X volume (120µl) of bead solution was added to each library and mixed thoroughly by pipetting. The strip tubes containing the mixed library and SpeedBeads were then placed on a magnet plate until the solution was clear. Then, whilst still on the magnet the supernatant was removed, taking care not to disturb the bead pellet. To wash the beads 80% filtered ethanol was then added to the strip tubes until the bead pellet was covered before being removed. This wash step was then repeated. Bead pellets were then air-dried (approximately 5 minutes), taking care not to allow over-drying. Once dry and removed from the magnet the cleaned library was then eluted in 30µl EBT heated to 37°C, mixed thoroughly by pipetting. Replacing this mix on the magnet plate and removing the supernatant produced the clean library which was then transferred to new strip tubes.

To deduce the appropriate number of cycles for the indexing PCR step a qPCR reaction was prepared. A volume of 2µl of 10X diluted library was added to a qPCR plate. To each library was added 10µl 2X KAPA SYBR qPCR mastermix, 5.32µl molecular grade H_2O, 0.34µl forward primer (IL amp P5 10µM) and 0.34µl reverse primer (IL amp P7 10µM). This reaction was prepared on ice and then run on a Bio-Rad CFX Connect™ Real-Time PCR detection system using a temperature profile as follows, 95°C denaturation for 3 minutes, 40 cycles of 94°C for 30 seconds, 60°C annealing for 20 seconds and 72°C extension for 20 seconds and a final extension of 72°C for 7 minutes. Appropriate number of cycles for indexing was determined as the number of cycles after which an asymptotic plane of amplification was approached.

Following qPCR, libraries were grouped according to the number of indexing PCR cycles required (10, 12 or 15 cycles). Each reaction was prepared by mixing, in strip tubes, 23µl of microsatellite library, 5µl 5X Phusion® buffer, 0.4µl dNTP, 1µl P5 primer, 1µl Phusion® High-Fidelity DNA polymerase (New England Biosystems), 18.6µl H_2O and 1µl of the appropriate P7 index primer for that library (see Table 3.1). Indexed libraries were then cleaned using SpeedBeads™ as described previously.

Prepared microsatellite libraries were then subject to a bait capture protocol using myBaits™. This protocol is outlined broadly in the following paragraphs.

Before providing a detailed protocol the components are outlined below. Hybridisation reagents are as follows: 19.46X SSPE & 13.5mM EDTA (Hyb N), 10%SDS (Hyb S), 50X Denhardt's solution (Hyb D) and RNAsecure™ (Thermo Fisher)(Hyb R). Blockers are Human Cot-1 DNA 1µg/µl (Block C), Salmon sperm DNA 1µg/µl (Block O) and adapter specific blocking oligos 1µg/µl (Block A). The binding buffer was 1M NaCl, 10mM Tris-HCl (pH 7.5) and 1mM EDTA. Wash buffer

was 0.1X SSC, 0.1% SDS and 1mM EDTA. Cleaning beads were MyOne C1 Dynabeads by Thermo Fisher. Baits are 100ng/µl ssRNA custom oligos.

First, we constructed a hybridisation mix consisting of 9.25µl Hyb N, 3.5µl Hyb D, 0.5µl Hyb S, 1.25µl Hyb R and 5.5µl of Baits per capture reaction to be performed. The hybridisation mix was then incubated at 60°C for ten minutes. After resting the incubated hybridisation mix (HYBs) at room temperature for 5 minutes, 18.5µl of mix was aliquoted to a 0.2mL tube for each library to be subject to capture.

In a similar fashion a blocker mix was prepared with 0.5µl Block A, 2.5µl Block C and 2.5µl Block O per capture reaction. Then, 5µl of this blocker mix was pipetted in to a 0.2mL tube along with 7µl of microsatellite library (now referred to as LIB) which is then heated to 95°C for five minutes followed by five minutes at the appropriate hybridisation temperature for five minutes. After this 18µl of HYBs, preheated to the hybridisation temperature, is then added to each LIB, carefully pipetting to mix. This reaction is then held at the hybridisation time for 16-24 hours.

Following hybridisation libraries were subjected to a bead clean. Beads were prewashed three times in Binding Buffer. Bead captured libraries were washed a total of three times in Wash Buffer. Cleaned libraries were resuspended in 30µl of 10mM Tris-Cl, 0.05% TWEEN-20 solution. Clean hybridised libraries were amplified in a 50µl reaction (5µl H2O, 25µl 2X KAPA HiFi HotStart ReadyMix, 2.5µl forward primer, 2.5µl reverse primer and 15µl of library) with a temperature profile as follows, 98°C denaturation for 2 minutes, 14 cycles of 98°C for 20 seconds, 60°C annealing for 30 seconds and 72°C extension for 30 seconds and a final extension of 72°C for 5 minutes. Amplified libraries were quantified with Qubit and qPCR before being pooled ready for sequencing across two lanes of sequencing.

Final microsatellite capture libraries were submitted for sequencing at DBS Genomics, Durham University at a final concentration of 4nM and run on an Illumina HiSeq 2500 using a Rapid run mode.

Raw Illumina sequence data for bait-captured microsatellites were demultiplexed with a custom Python script. The sample with the largest number of captured sequences (Sv33) was then used to create a *de novo* assembly using Trinity assembler (Grabherr *et al*., 2011). All other sample sequence data was then aligned against this assembly using Bowtie2 (Langmead and Salzberg, 2012). Resultant .sam files were converted to .bam files for later use using samtools (Li *et al*., 2009). Microsatellites within the Sv33 sequence assembly were identified using the MISA (MIcroSAtellite identification tool) online web server (Beier *et al*., 2017). File-size curtailing was required but this still allowed the identification of 3000+ microsatellite sequences. Genotyping was completed using PSR (Cantarella and D'Agostino, 2015).

Appendix IV.II – Results

Assembly of reference data using Trinity for sample Sv33 produced 3445 reads containing microsatellite sequences. Unfortunately, the PSR pipeline revealed that most of these reads were unique and only 34 identified microsatellites (Table AV.2) had a read depth of greater than two (maximum read depth was 12). Within these microsatellites there was a mix of mononucleotide (n=24), dinucleotide (n=8) and trinucleotide (n=2) repeats. The PSR pipeline further revealed that for each of these 34 microsatellites coverage across the study genotypes was extremely low and no microsatellite appeared within greater than two further individuals, despite high number of reads retained (mean = 506,069 reads). Thus, despite attempts with multiple software packages, the underlying data structure prevented further analysis.

Table AV.2: Retrieved microsatellite (SSR) sequences following PSR analysis. Further analysis was discontinued due to insufficient read coverage and low population presence.

Assembly Sequence ID	SSR Number	SSR Motif	Start Location	Stop Location	Repeat Unit	Total Reads
DN61	1	(T)18	160	177	18	3
DN73	2	(TG)6	818	829	6	5
DN76	3	(T)25	44	68	16	2
DN437	4	(A)15	241	255	15	2
DN433	5	(A)24	337	360	8	2
DN8821	6	(T)11	167	177	11	2
DN245	7	(AC)14	166	193	4	2
DN14009	8	(A)11	196	206	8	2
DN12712	9	(T)11	16	26	11	2
DN11002	10	(A)16	446	461	8	2
DN1411	11	(A)20	318	337	20	2
DN19136	12	(T)10	166	175	10	2
DN6403	13	(T)12	166	177	11	4
DN165	14	(AC)16	70	101	16	11
DN165	15	(GT)16	57	88	16	5
DN150	16	(GT)10	161	180	10	2
DN602	17	(CA)12	148	171	13	2
DN11353	18	(GAG)5	221	235	5	2
DN7188	19	(T)10	167	176	10	3
DN983	20	(T)11	29	39	11	2
DN15283	21	(T)12	166	177	10	5
DN15283	22	(T)12	166	177	12	3
DN15291	23	(A)12	227	238	12	2
DN16635	24	(T)11	355	365	10	7
DN16635	25	(T)11	355	365	11	5
DN1011	26	(T)13	171	183	13	2
DN379	27	(CA)15	330	359	15	2
DN308	28	(T)20	37	56	20	2
DN15698	29	(A)12	217	228	12	2
DN5965	30	(T)15	163	177	15	2
DN5131	31	(AAC)8	216	239	8	2
DN718	32	(T)16	176	191	16	2
DN2222	33	(TG)12	139	162	4	2
DN16470	34	(T)12	210	221	12	2

Appendix IV.III – Lessons learnt and future approaches

This approach attempted to create a large microsatellite dataset through bait capture to examine in greater detail the suggestion of genetic differentiation in *S. viridensis* observed across the Italian peninsula in Milana *et al* (2014). Ultimately this was not successful but there are lessons to be learnt from this for future studies. Fundamentally this technique proved unsuccessful due to the low levels of shared coverage of any given microsatellite across individuals, despite an average of over half a million reads retained per individual. This may have arisen due to insufficient specificity in the custom baits utilised. Future attempts could attempt using more specific baits, akin to short primers, to increase the likelihood of uniform capture across samples. Furthermore, as whole genome sequencing becomes increasingly financially accessible for non-model organisms a barracuda sp. genome could be used to direct the bait design to increase the potential capture yield. Attempts were made in this study to make use of the Cod *Gadus morhua* (GenBank Accession GCA_902167405.1) genome in this way but the significant evolutionary distance between the two species (most recent common ancestor approximately 145 million years ago (Hughes *et al*., 2018; Near *et al*., 2012)) resulted in the approach being suspended at an early stage.

Utilising next generation sequencing for microsatellite enriched libraries to discover suitable targets (sometimes several hundred) for PCR amplification is now relatively common (Fougat *et al*., 2014; Zalapa *et al*., 2012). The technique attempted here builds on this to produce libraries containing thousands of microsatellites multiplexed across many individuals and is certainly worth pursuing for future studies.

Regardless of the outcome of this ambitious approach, it is not my belief that future attempts would fail, provided they learnt from the observations included here. It is for that reason it was felt prudent to include a record of this approach in this thesis, with an Appendix ensuring it does not detract from the ultimately successful elements of Chapter 3.

Appendix V – Stable isotope data for *T. truncatus*

Sample ID	d15N	d13C	Sea	Long	Lat	Tissue	Storage
CL541	13.88	-15.67	Adriatic	12.3922	44.22333	Skin	Frozen
CL546	13.83	-16.46	Adriatic	12.3063	44.45984	Unk..	Lyophilised
CRO101	13.56	-14.17	Adriatic	14.47952	44.6057	Skin	DMSO
CRO18	11.36	-16.92	Adriatic	14.47952	44.6057	Muscle	Lyophilised
CRO24	13.64	-16.87	Adriatic	14.0436	44.8203	Skin	DMSO
CRO3	11.67	-15.99	Adriatic	14.0436	44.8203	Lung	Lyophilised
CRO35	14.55	-15.06	Adriatic	14.24428	44.63548	Skin	DMSO
CRO45	15.48	-15.25	Adriatic	15.2501	44.1002	Skin	Ethanol
CRO57	11.12	-16.88	Adriatic	14.8512	44.3544	Skin	DMSO
CRO74	10.94	-17.22	Adriatic	14.6254	43.6801	Skin	DMSO
MAR3	14.15	-16.12	Adriatic	12.8777	43.7601	Muscle	Frozen
RV3P	13.21	-16.73	Alborán	-6.46923	36.90454	Unk..	Unk..
RV7M	13.99	-16.60	Alborán	-5.52551	36.03065	Unk..	Unk..
RB26	16.61	-14.54	Alborán	-2.473	36.81382	Unk..	Unk..
RB35	15.05	-14.41	Alborán	-5.52551	36.03065	Skin	DMSO
AZ101	11.35	-17.55	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ103	10.82	-17.46	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ104	11.49	-17.98	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ105	11.82	-17.07	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ107	11.12	-19.13	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ2	13.92	-16.10	Atlantic	-28.5776	38.5348	Skin	Ethanol
AZ109	12.92	-17.85	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ3	13.95	-15.77	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ41	11.76	-17.08	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ45	10.00	-17.43	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ48	13.97	-15.60	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ54	10.61	-17.74	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ7	12.45	-16.00	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ8	14.59	-15.46	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ83	11.46	-18.14	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ84	11.44	-17.78	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ90	12.16	-17.57	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ91	14.00	-15.61	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ93	11.13	-17.54	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ97	12.02	-17.44	Atlantic	-28.5776	38.5348	Muscle	Ethanol
AZ99	11.15	-17.50	Atlantic	-28.5776	38.5348	Muscle	Ethanol
GRE15	10.51	-15.93	Greece	23.76472	38.80194	Skin	DMSO
GRE10	9.96	-15.76	Greece	23.375	38.975	Muscle	DMSO
GRE1	13.17	-16.91	Greece	20.6054	38.9324	Muscle	DMSO
25-G	15.65	-14.57	Cádiz	-6.52499	36.9119	Unk..	Unk..
31-G	15.83	-15.11	Cádiz	-6.52499	36.9119	Unk..	Unk..

34G	16.00	-14.04	Cádiz	-6.52499	36.9119	Unk..	Unk..
37G	16.40	-14.18	Cádiz	-6.52499	36.9119	Unk..	Unk..
42G	15.61	-14.20	Cádiz	-6.52499	36.9119	Unk..	Unk..
43G	15.37	-14.28	Cádiz	-6.52499	36.9119	Unk..	Unk..
44G	15.49	-14.58	Cádiz	-6.52499	36.9119	Unk..	Unk..
45G	14.95	-14.59	Cádiz	-6.52499	36.9119	Unk..	Unk..
47-G	16.08	-14.59	Cádiz	-6.52499	36.9119	Unk..	Unk..
48G	16.49	-12.89	Cádiz	-6.52499	36.9119	Unk..	Unk..
SIC01	11.11	-18.05	Sicily	12.64107	37.56873	Skin	DMSO
SIC02	9.78	-16.70	Sicily	12.64107	37.56873	Skin	DMSO
SIC03	9.45	-17.56	Sicily	12.64107	37.56873	Skin	DMSO
SIC05	10.07	-16.59	Sicily	12.64107	37.56873	Skin	DMSO
SIC06	10.08	-16.85	Sicily	12.64107	37.56873	Skin	DMSO
SIC09	11.51	-16.39	Sicily	12.64107	37.56873	Skin	DMSO
SIC12	10.25	-16.94	Sicily	12.64107	37.56873	Skin	DMSO
SIC13	10.59	-16.90	Sicily	12.64107	37.56873	Skin	DMSO
TUS13	14.04	-15.12	Tyrrhenian	10.9089	42.6983	Unk..	Lyophilised
TUS14	12.32	-16.68	Tyrrhenian	10.2299	42.8391	Muscle	Lyophilised
TUS16	13.10	-16.25	Tyrrhenian	10.2133	43.5439	Kidney	Lyophilised
TUS17	13.32	-16.22	Tyrrhenian	10.2133	43.5439	Liver	Lyophilised
TUS20	12.73	-16.25	Tyrrhenian	10.219	43.548	Muscle	Lyophilised
TUS27	13.68	-15.72	Tyrrhenian	10.2133	43.5439	Heart	Lyophilised
TUS29	12.39	-17.21	Tyrrhenian	10.2151	43.8981	Liver	Lyophilised
TUS30	13.24	-15.85	Tyrrhenian	10.9089	42.6983	Liver	Lyophilised
TUS31	13.14	-16.62	Tyrrhenian	10.2133	43.5439	Liver	Lyophilised
TUS5	10.68	-17.49	Tyrrhenian	10.2051	42.8186	Muscle	DMSO
VAL1	15.05	-16.08	Valencia	-0.32241	39.47416	Skin	Frozen
VAL2	14.49	-16.40	Valencia	-0.32241	39.47416	Skin	Frozen
VAL3	13.89	-16.08	Valencia	-0.32241	39.47416	Skin	Frozen
VAL4	12.91	-16.07	Valencia	-0.32241	39.47416	Skin	Frozen
VAL6	13.88	-16.10	Valencia	-0.32241	39.47416	Skin	Frozen
VAL7	15.47	-13.88	Valencia	-0.32241	39.47416	Skin	Frozen
VAL9	14.37	-16.24	Valencia	-0.32241	39.47416	Skin	Frozen
VAL10	14.94	-14.29	Valencia	-0.32241	39.47416	Skin	Frozen

Appendix VI – Mantel test data visualisation for *S. viridensis*

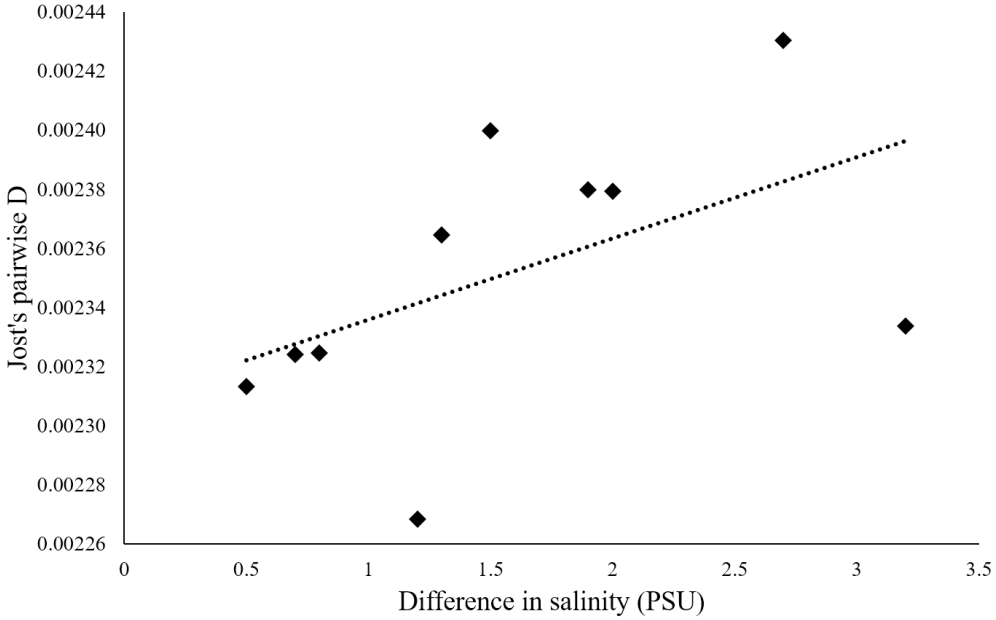


Figure IV.1: Genetic distance (Jost's pairwise D) vs difference in local salinity for *Sphyraena viridensis* populations as used for Mantel testing. Dotted line is a fitted line and does not represent a regression.

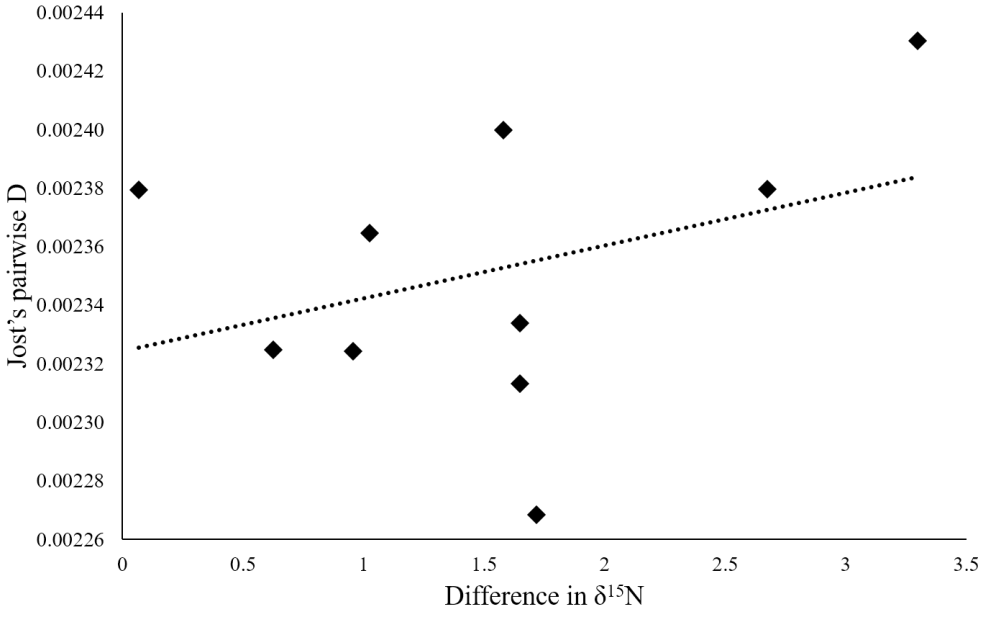


Figure IV.2: Genetic distance (Jost's pairwise D) vs difference in $\delta^{15}N$ for *Sphyraena viridensis* populations as used for Mantel testing. Dotted line is a fitted line and does not represent a regression.

Appendix VII – Migration rates for *T. truncatus* ecotypes

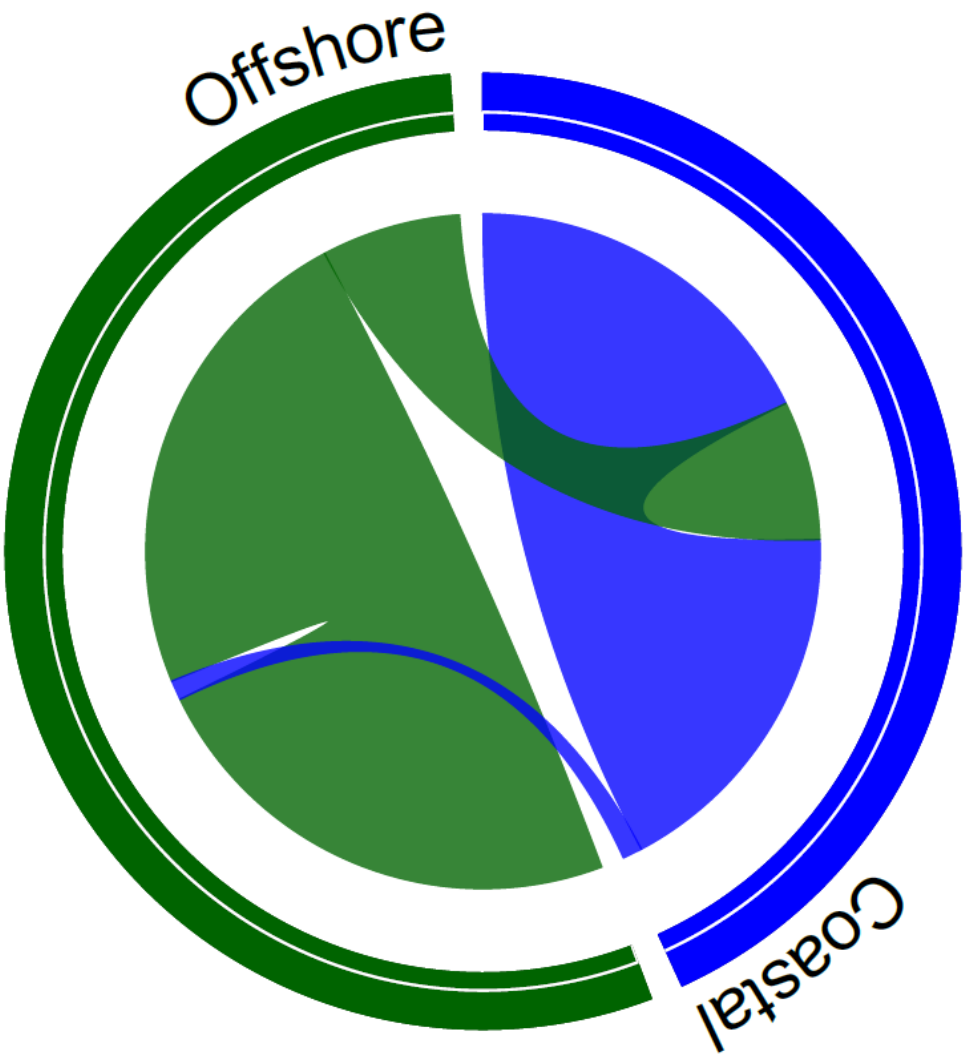
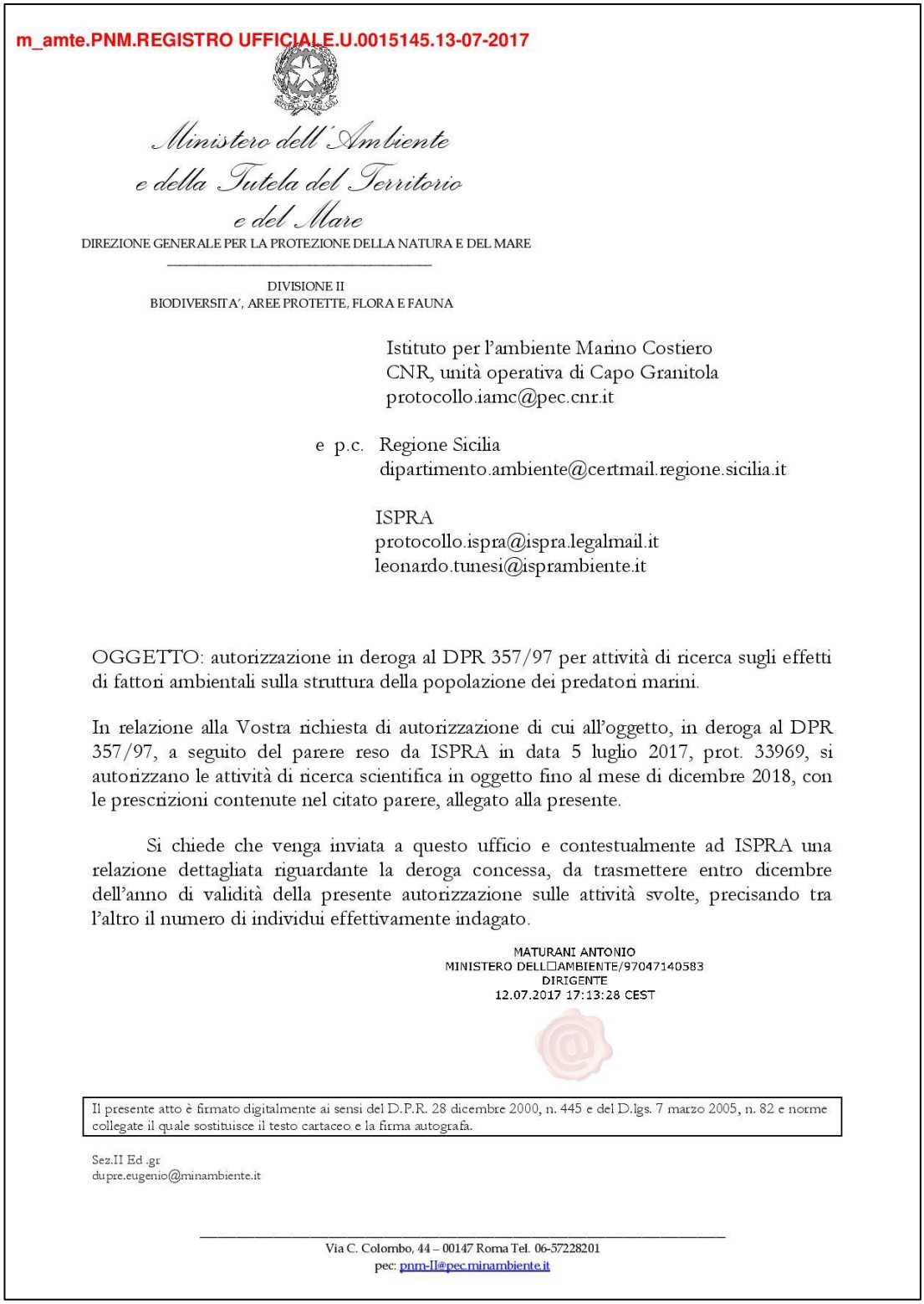


Figure VII.1: Circosplot showing migration rates between the Offshore and Coastal populations as calculated by BayesAss3-SNPs without potential misidentified samples. Figure migration values are found in Table VII.1.

Table VII.1: Matrix of inferred (posterior mean) migration rates per generation. Values in brackets represent migration as a proportion of population size. Migration rates are in the direction column to row, thus a slightly higher migration rate from the Offshore to the Coastal ecotype population was observed.

Population	Coastal	Offshore
Coastal	0.7271 (0.0305)	0.2729 (0.0305)
Offshore	0.0384 (0.0248)	0.9616 (0.0248)

Appendix VIII – Permits and permissions



m_amte.PNM.REGISTRO UFFICIALE.U.0015145.13-07-2017

Ministero dell'Ambiente
e della Tutela del Territorio
e del Mare

DIREZIONE GENERALE PER LA PROTEZIONE DELLA NATURA E DEL MARE

DIVISIONE II
BIODIVERSITA', AREE PROTETTE, FLORA E FAUNA

Istituto per l'ambiente Marino Costiero
CNR, unità operativa di Capo Granitola
protocollo.iamc@pec.cnr.it

e p.c. Regione Sicilia
dipartimento.ambiente@certmail.regione.sicilia.it

ISPRA
protocollo.ispra@ispra.legalmail.it
leonardo.tunesi@isprambiente.it

OGGETTO: autorizzazione in deroga al DPR 357/97 per attività di ricerca sugli effetti di fattori ambientali sulla struttura della popolazione dei predatori marini.

In relazione alla Vostra richiesta di autorizzazione di cui all'oggetto, in deroga al DPR 357/97, a seguito del parere reso da ISPRA in data 5 luglio 2017, prot. 33969, si autorizzano le attività di ricerca scientifica in oggetto fino al mese di dicembre 2018, con le prescrizioni contenute nel citato parere, allegato alla presente.

Si chiede che venga inviata a questo ufficio e contestualmente ad ISPRA una relazione dettagliata riguardante la deroga concessa, da trasmettere entro dicembre dell'anno di validità della presente autorizzazione sulle attività svolte, precisando tra l'altro il numero di individui effettivamente indagato.

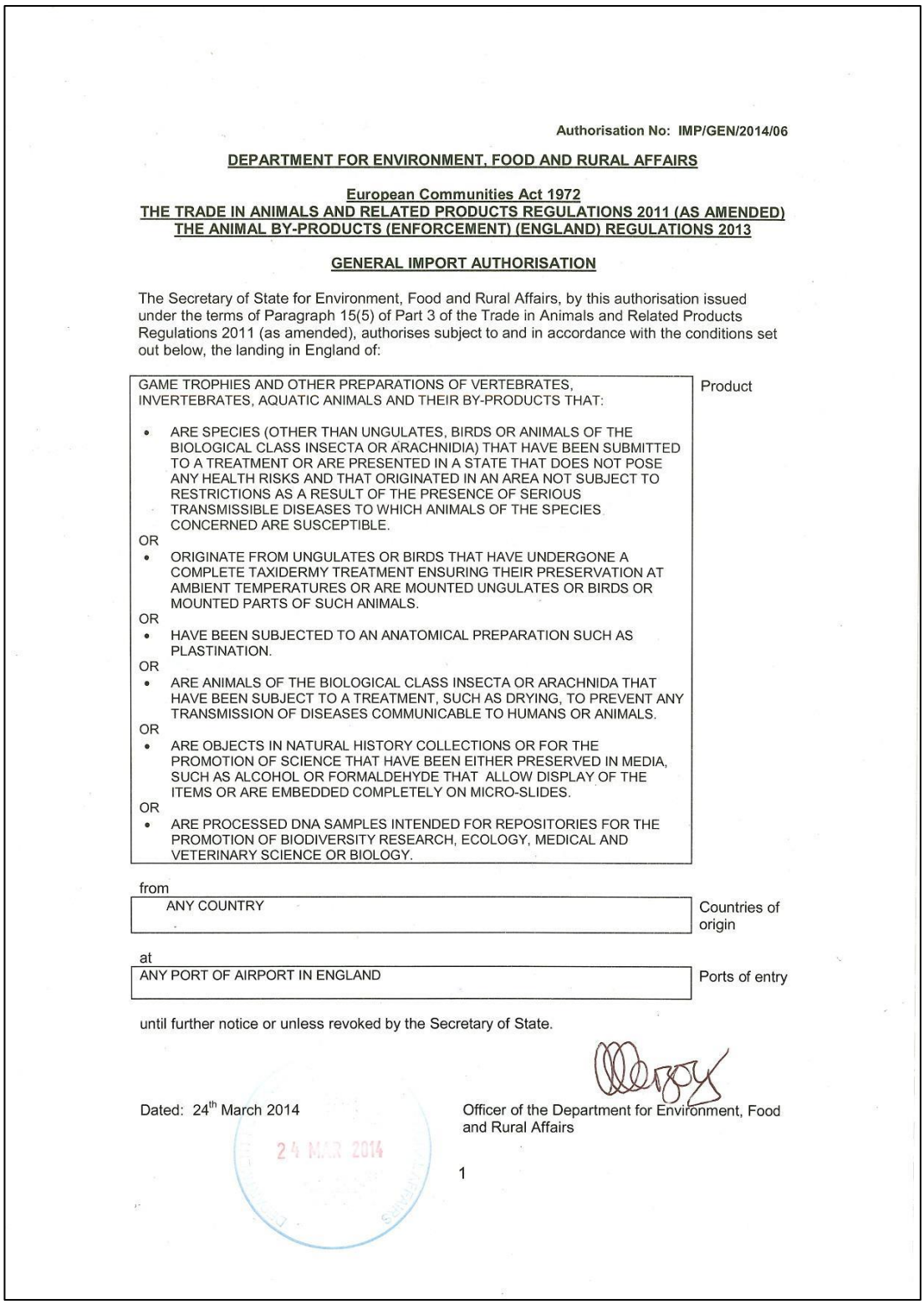
MATURANI ANTONIO
MINISTERO DELL□AMBIENTE/97047140583
DIRIGENTE
12.07.2017 17:13:28 CEST

Il presente atto è firmato digitalmente ai sensi del D.P.R. 28 dicembre 2000, n. 445 e del D.lgs. 7 marzo 2005, n. 82 e norme collegate il quale sostituisce il testo cartaceo e la firma autografa.

Sez.II Ed .gr
dupre.eugenio@minambiente.it

Via C. Colombo, 44 – 00147 Roma Tel. 06-57228201
pec: pnm-II@pec.minambiente.it

Figure VIII.1: Permit for biopsy sampling (No. 33969) from *Ministero dell'Ambiente e della Tutela del Territorio e del Mare*.



Authorisation No: IMP/GEN/2014/06

DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

European Communities Act 1972
THE TRADE IN ANIMALS AND RELATED PRODUCTS REGULATIONS 2011 (AS AMENDED)
THE ANIMAL BY-PRODUCTS (ENFORCEMENT) (ENGLAND) REGULATIONS 2013

GENERAL IMPORT AUTHORISATION

The Secretary of State for Environment, Food and Rural Affairs, by this authorisation issued under the terms of Paragraph 15(5) of Part 3 of the Trade in Animals and Related Products Regulations 2011 (as amended), authorises subject to and in accordance with the conditions set out below, the landing in England of:

GAME TROPHIES AND OTHER PREPARATIONS OF VERTEBRATES, INVERTEBRATES, AQUATIC ANIMALS AND THEIR BY-PRODUCTS THAT: • ARE SPECIES (OTHER THAN UNGULATES, BIRDS OR ANIMALS OF THE BIOLOGICAL CLASS INSECTA OR ARACHNIDIA) THAT HAVE BEEN SUBMITTED TO A TREATMENT OR ARE PRESENTED IN A STATE THAT DOES NOT POSE ANY HEALTH RISKS AND THAT ORIGINATED IN AN AREA NOT SUBJECT TO RESTRICTIONS AS A RESULT OF THE PRESENCE OF SERIOUS TRANSMISSIBLE DISEASES TO WHICH ANIMALS OF THE SPECIES CONCERNED ARE SUSCEPTIBLE. OR • ORIGINATE FROM UNGULATES OR BIRDS THAT HAVE UNDERGONE A COMPLETE TAXIDERMY TREATMENT ENSURING THEIR PRESERVATION AT AMBIENT TEMPERATURES OR ARE MOUNTED UNGULATES OR BIRDS OR MOUNTED PARTS OF SUCH ANIMALS. OR • HAVE BEEN SUBJECTED TO AN ANATOMICAL PREPARATION SUCH AS PLASTINATION. OR • ARE ANIMALS OF THE BIOLOGICAL CLASS INSECTA OR ARACHNIDA THAT HAVE BEEN SUBJECT TO A TREATMENT, SUCH AS DRYING, TO PREVENT ANY TRANSMISSION OF DISEASES COMMUNICABLE TO HUMANS OR ANIMALS. OR • ARE OBJECTS IN NATURAL HISTORY COLLECTIONS OR FOR THE PROMOTION OF SCIENCE THAT HAVE BEEN EITHER PRESERVED IN MEDIA, SUCH AS ALCOHOL OR FORMALDEHYDE THAT ALLOW DISPLAY OF THE ITEMS OR ARE EMBEDDED COMPLETELY ON MICRO-SLIDES. OR • ARE PROCESSED DNA SAMPLES INTENDED FOR REPOSITORIES FOR THE PROMOTION OF BIODIVERSITY RESEARCH, ECOLOGY, MEDICAL AND VETERINARY SCIENCE OR BIOLOGY.	Product
from ANY COUNTRY	Countries of origin
at ANY PORT OF AIRPORT IN ENGLAND	Ports of entry

until further notice or unless revoked by the Secretary of State.

Dated: 24th March 2014

Officer of the Department for Environment, Food and Rural Affairs

24 MAR 2014

1

Figure VIII.2: Import authorisation from DEFRA as permitted by the trade in animals and related products regulations 2011.